

Can Confucius excuse poor creativity?

Respect for authority is not conducive to the sceptical thinking that accompanies originality. But Korea's strong Confucian values are less of an impediment to the country's scientific progress than other, more commonplace obstacles.

KOREA, so Koreans say, is one of the few Asian countries in which students bow to their professors. That, those professors will tell you, is a symptom of Confucian traditions of respect for elders and for scholarship. Probably as a by-product of the country's historical need to preserve its national and cultural identity in the looming presence of China and Japan, the Confucian tradition remains stronger in the Republic of Korea than in any other country.

That tradition is one of the cultural strengths that has helped the nation make extraordinary strides in industrial power in recent decades. As with other Asian states, autocracy from the top and respect from below, combined with sheer unflagging hard work, has allowed Korea to extract its sizeable share of wealth from the global economy. And this at a time when the nation has been on a perpetual war footing against bellicose cousins to the north.

Now Korea needs that purpose and resilience as much as ever. Other states are supplying cheaper labour — Korean wages have doubled over the last few years — while export revenue in at least one of its key industrial sectors, semiconductor circuits, appears wobbly. The country has met the challenge of moving to higher added-value products only to a limited extent. The country's industrial giants are widely perceived as overly protected and slow to take on new challenges. That is probably too much of a generalization. Nevertheless, although industry spends much money on R&D, senior figures speak of a need for more in the way of fundamental technological innovation.

What of science amidst all of this? To its enormous credit, the country has established an academic science base virtually from scratch over the last two to three decades. Although there are over 100 national and private universities, only fifteen or so could be said to constitute part of the science research base, and only a handful of those are now perceived to be foreseeably capable of establishing themselves firmly in the international league. The influence of the United States in this extraordinarily rapid development cannot be overstated. It is no coincidence that the university that achieved the highest ranking in science and technology in a recent survey, the Pohang University of Science and Technology, was set up — as recently as nine years ago — with a mass import of Koreans from excellent US institutions.

Of course it takes researchers in any country several years to build up a world-class laboratory. But Koreans, ever self-critical, rightly fret about other factors that limit their scientific achievements, and their apparent lack of creativity in particular. Take schools, for instance. Parents spend as much as 50% of their income on high school education in order to get their young into prestigious universities, on which subsequent careers depend too heavily. That process leaves young university students crammed to the brim with knowledge but with little experience of imaginative and creative thinking. Those that do not, exhausted, put their feet up, but instead wish to take science seriously, find themselves in a university system in which a Confucian respect for authority and the need to get through the next exams are paramount.

All that, say young and not-so-young Korean academics, seriously undermines their capacity for creativity. And those who have returned from the creative hot-houses in the West? As soon as they return, it is said with a rueful laugh, they become Koreans again.

Maybe there is hope to be had in the fact that Confucian standards are slipping. Not often nowadays will young researchers wait for their laboratory heads to go home before they themselves pack up for the day. But to place much emphasis on such change would be misguided. The prime needs of Korean researchers are those that their Western counterparts would find all too familiar — less bureaucracy and less short-termism — and, in some disciplines, better supplies of equipment and more ready access to consumables from abroad.

Distractions

The burdens of bureaucracy arise most obviously in the funding of research. Grants are awarded for periods of up to three years (and in a new scheme some will be awarded for up to five) but young scientists find themselves obliged to produce reports and even, some say, publications every year. That is too onerous a burden on people engaged in the medium-term development of centres of high-quality research. The various ministries that support university research should take steps to diminish their administrative zeal. Senior academics should ensure that their younger staff are not mopping up too much of the paperwork. At the same time, staff-student ratios should be increased to reduce teaching burdens.

Short termism stems from the top. Korea's Economic Planning Board is responsible for overall policy, and is widely seen to be dominated by economists and industrialists with blinkered views as to the purposes of research. That perspective infects too many of the country's sources of research funding. True, a greater emphasis on basic research is apparent in recent policy developments, but much more will be needed in order to fulfil at least one of the next aims of the Korean government: to develop a strong scientific base in the life sciences that will stimulate associated industries.

Despite the problems, this is no bad time to be a Korean scientist. Research budgets from all sources are set to increase faster than inflation, science is receiving priority attention from the government, and science and technology — and the skills that go with them — are widely perceived to be essential for Korea's next phase of development. Increased competitiveness and mobility in appointments, and making foreign researchers more welcome with practical support, would help the country move even faster to the excellence it seeks.

So how to boost scientific creativity? Confucian values notwithstanding, receptivity and support for new ideas is what counts. Korea already has its centres of excellence. Now it needs to give its brightest researchers ample time to think, to be stimulated by contact with researchers at the leading edge overseas, and — gradually — to bloom. What better defining quality for the next decade of the country's scientific history? And what better seeds of technological and economic innovation in the decades to follow? □



Lost Mars mission thrusts Russian space science further into crisis

Washington, Moscow & London. Despite predictions of an imminent collapse, most observers agree that Russia's space science establishment is likely to survive last week's loss of the Mars 96 spacecraft. But survival will probably be at a reduced level. And Russian space scientists will almost certainly never regain the leading role they once played in planetary exploration.

In fact, the loss of the spacecraft, when combined with the apparent failure of the Proton launch system and worsening problems with Russian participation in the international space station, appears to have brought the nation's space programme to a point of crisis.

Mars 96 cost Russia \$122 million and collaborators from 20 countries another \$180 million, according to figures released in Moscow on Monday. The spacecraft re-entered the atmosphere after an upper stage boosted by a Proton rocket failed to work properly. Russian space scientists and engineers had been working on the project for nearly a decade, and its launch had been postponed for two years from the originally planned date.

While a hastily formed committee began investigating the technical causes of the failure, dejected scientists at the Russian space research institute (IKI), which sponsored the mission, began studying options for reviving the mission. One idea, according to Thomas Duxbury of the US Jet Propulsion Laboratory, a participating scientist in Mars 96 who attended the launch, would be to re-fly many of the same instruments on a smaller spacecraft, since most have spares already built. The United States launched a similar mission this month, called the Mars Global Surveyor, to recover most of the data from the lost Mars Observer spacecraft.

Planetary scientists were encouraged by comments from officials of the Russian Academy of Sciences, who said this week that they would continue to support Russia's planetary exploration programme, even if it meant shifting resources from planned astrophysics missions to pay for it. But a recovery flight — if it materializes — will not happen quickly. Yuri Koptev, director general of the Russian space agency, says there will be no attempt to reach Mars again for at least five years.

A recovery flight also would have competition. Both Russia and the US National Aeronautics and Space Administration (NASA) have already made preliminary plans for a joint mission in 2001, with Russia providing a surface rover to complement an

American orbiter. Some of the Mars 96 lander instruments could possibly be shifted to the rover, said Duxbury.

A Russian instrument is also scheduled to fly on a US Mars lander due for launch in 1998. An international Mars exploration working group will meet in Florida next month, following the launch of NASA's Mars Pathfinder spacecraft, to consider these and other plans for future Mars missions.

Initial reactions from European and American scientists suggest that Russia will remain a welcome partner in such projects. Western scientists admit to being upset at the lack of rigorous hardware testing that preceded the Russian launch. According to Duxbury, however, the level of pre-launch testing for Mars 96 was much higher than for the last Russian attempt at Mars in the 1980s.

Despite the Mars 96 loss — and the fact that all but one Russian Mars mission since the 1960s has failed — many international investigators say they would still continue to work with IKI in the future, and expect the Russian space science programme to survive. "Space science is too big an industry in Russia to be affected in any major way," says Jean André Sauvaud, a Mars 96 researcher with the French Centre National de la Recherche Scientifique (CNRS).

But money remains a worsening problem. The contractors who built different parts of the Mars 96 spacecraft reported after last weekend's launch failure that they had not been paid in months. "At least one reason for this greatest misfortune in our Martian programme is evident," says Alexander Alferov, scientific secretary of the Russian Academy of Sciences Council for the Cosmos. "Talented people are leaving space research teams because of poor financing."

Another negative result of the Mars 96 failure may be a loss of revenue from the

country's commercial Proton sales. The rocket, which is normally highly reliable, has now suffered two recent upper-stage failures, and this may well drive away some potential customers.

The country's manned spaceflight programme, which represents by far the larger share of space funding, is already on the verge of financial disaster. NASA officials

flew to Moscow this week for a series of urgent meetings with their Russian counterparts aimed at resolving schedule and funding problems that are related to the international space station.

Most observers now expect the schedule for Russian delivery of station components to slip, and NASA is likely to face renewed criticism in the US Congress for its reliance on Russian hardware. Furthermore, Congress is keen to see hard currency flowing.

Marcia Smith, a Russian space programme expert with the US Congressional Research Service,

warns that all these problems should not necessarily be lumped together. The Mars 96 failure, if it turns out to be due to the rocket upper stage, should be combined with other recent international launch failures like the European Ariane and the US Pegasus. It may not be proof, says Smith, that financial problems have eroded the quality of Russian science missions, as the spacecraft had no chance to prove itself.

At the same time, even with a commitment from the Academy of Sciences, Russia's space scientists, left with no mission to work on until 2001, will be struggling to stay afloat. The IKI itself may survive, but it may lose staff. Even if the rocket, and not the spacecraft, is to blame for the Mars 96 disaster, says Smith, "it just so happens it was carrying the one spacecraft on which the whole of the Russian space science community was riding".

**Tony Reichhardt,
Carl Levitin & Ehsan Masood**

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Up in smoke: After a perfect lift-off, failure of the launcher's upper stage led to disaster.

US plans for weapons labs 'have ignored alternatives'

Washington. Environmental and disarmament groups say they will take legal action to force the US Department of Energy (DoE) to revise a blueprint for the future of its nuclear weapons complex that was released in Washington last week. They argue that alternatives — including reducing the size of the complex — have not been fully explored.

The five-volume document is known as the Stockpile Stewardship and Management Programmatic Environmental Impact Statement. It essentially backs decisions already announced by DoE. These include building the National Ignition Facility, a \$1.1-billion megalaser for weapons physics experiments, at Lawrence Livermore National Laboratory in California and establishing a capability for manufacturing plutonium weapon 'pits' at Los Alamos National Laboratory, New Mexico.

The document was printed in September, but was released only last week, leading to suggestions that publication was delayed until after the US elections. DoE officials deny this, arguing that the extra time was needed to respond to public comments and to consider the possibility of lawsuits.

According to Victor Reis, assistant secretary for defence programmes, the agency is confident it can withstand a legal challenge. But he accepts that an extended court fight could cause delays in implementing the \$40-billion, 10-year plan. "We feel comfortable we've covered all the issues [that are required under the National Environmental Policy Act]," Reis says. "We feel we are in a good position if we are sued."

But the Military Production Network (MPN), an umbrella group of environmental and disarmament organizations, says the analysis has failed properly to consider alternatives, as required under the act. More than 100 such groups threatened to sue DoE last May, after release of the draft impact

statement, and the agency's proposed course of action did not change in the final version of the document. MPN says a lawsuit is likely to be brought by the end of the year.

Christopher Paine, senior research associate with the Natural Resources Defense Council, the group that plans to lead the litigation, argues that, to meet the act's requirements, the department must consider alternatives, such as reducing the size of the network of weapons laboratories. Only last year President Bill Clinton ordered continuation of the present three-lab structure.

The consolidation alternative should especially have been explored in view of last year's recommendations from a DoE advisory panel, the Galvin Task Force, which proposed phasing out the weapons programme at Livermore, adds Daniel Kerlinksky, president of Physicians for Social Responsibility of New Mexico, and a Galvin panel member.

But Steve Guidice, assistant manager for energy science and technology at DoE's Albuquerque Operations Office, the official in charge of the impact statement, replies that the department did not consider reducing the size of the laboratory complex to be a "reasonable" alternative within the meaning of the act. This is particularly so given the department's need for greater quantities of physics data to guarantee a continued safe and reliable stockpile without testing.

Paine complains that the DoE has arbitrarily excluded from examination many of the components of its existing stockpile stewardship programme, either by classifying them as part of its 'no-action' alternative, or defining them as 'next-generation' facilities.

But Guidice says that plans for the latter facilities, which include an Advanced Hydrotest Facility and a High Explosive Pulsed Power Facility, were considered "not mature enough".

David Kramer

Greater exchange of scientists backed for Asia-Pacific

Seoul. Governments in the Asia-Pacific region have agreed to encourage mobility and exchange of scientists between their countries, and to stimulate creativity by promoting an appropriate social environment for the advancement of science and technology.

The agreement was reached by science ministers attending the second Asia-Pacific Economic Cooperation (APEC) forum in Seoul last week. APEC, which held its first forum for science ministers in Beijing last October, brings together a number of Asian countries, together with Australia, New Zealand, Chile, Canada, Mexico and the United States.

The meeting did not spell out how these goals are to be achieved. But the Seoul declaration, signed by 18 member countries, will give science ministers a useful tool to lobby for programmes to fit these aims.

The exchange of scientists is at present limited between many of the Asian countries in APEC, which include China, Korea, Japan, Taiwan, Hong Kong, Brunei, Thailand, Singapore, Malaysia, the Philippines, Indonesia, and Papua New Guinea. The ministers recommended a region-wide study of the existing obstacles to exchange.

The declaration also calls for greater sharing of national facilities, and increasing the flow of information between member countries through, for example, computer networks and web sites on the Internet. Some steps to encourage information flow have already been taken following the Beijing conference. For example, the APEC S&T Web, a web site that puts together a Japan-Korea project on information flow and Australia's APEC web site, was demonstrated at the meeting.

Korean officials announced plans for an Advanced Science and Technology Network (ASTN) that includes a research manpower training programme for researchers of developing economies in APEC. The network will be centred on the Korea Advanced Institute of Science and Technology (KAIST) in Taejeon science city, and will open next September.

Other elements are a postdoctoral research programme for young scientists; the sharing of advanced research facilities, for example at KAIST, Pohang University of Science and Technology in South Korea, and institutions in Japan, such as the Institute of Physical and Chemical Research; and an APEC science and technology information network. The ASTN secretariat will be based at KAIST, and the Korean government aims to have it fully operational by June 1998. David Swinbanks

Universities approve hospital merger

San Francisco. Merger of the hospitals of Stanford University and of the University of California in San Francisco (UCSF) was approved last week by the authorities at both universities.

The two schools will continue to operate independent research laboratories and medical schools, but are likely to carry out more collaborative projects, according to Gerhard Casper, president of Stanford.

Each medical school is contributing \$8.5 million to help start up a nonprofit corporation, UCSF Stanford Health Care, which will run the two Stanford and two

UCSF hospitals. The controversial merger, which will combine the University of California's public assets with the private Stanford enterprise, emerged out of difficulties both schools have experienced in the highly competitive 'managed care' environment in California.

University officials say that the merged schools will remain competitive and able to finance their extensive research operations. The plan must still undergo legal scrutiny by the state attorney general and will face strong opposition from unions representing UCSF's 4,940 employees.

Sally Lehrman

Split over 'privatization' of UK observatories

London. Plans for 'privatizing' the management of Britain's overseas observatories and telescope development activities have apparently been delayed by sharp differences at top levels of government over how much of the costs involved should be taken out of the science budget.

Scientists are concerned at the prospect of having to cut back on research programmes to cover an estimated £12 million (US\$20 million) in pension commitments to observatory staff that would have to be included in any privatization package. Whether this should happen is reported to be a heated point of discussion over next year's budget for the whole Department of Trade and Industry (DTI), due to be announced next week as part of the government's budget for next year.

Plans to invite bids from private organizations and others for managing the Royal Greenwich Observatory and the Royal Observatory of Edinburgh, as well as telescopes in Hawaii and the Canary islands, were first announced by the government earlier this year (see *Nature* 381, 3; 1996).

Interested bodies include the University of Cambridge, where the Greenwich observatory is now based, and the University of Edinburgh, as well as the Central Laboratory of the Research Councils (CLRC) — previously the Daresbury and Rutherford Appleton Laboratories.

Invitations for tender had been due to be sent out at the end of October. But this was delayed last week for a second time. No official reason has been given. But it is widely

rumoured that a large stumbling block is the question of the pensions liability.

Indeed, the whole issue of who should pay for the costs of pension transfers associated with the privatization of research institutes has become a bone of contention between the Treasury, worried about extra financial commitments, and ministers keen to see the management of Britain's science opened up to market forces.

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Looking for an answer: Heseltine (left) and Waldegrave (right) are said to disagree over paying for pensions.

The latter is being actively pursued by Michael Heseltine, deputy prime minister, who, in his previous position as President of the Board of Trade, was responsible for privatizing bodies such as the Warren Spring Laboratory and the National Physical Laboratory (see *Nature* 376, 206; 1995).

Ironically, the Treasury's position is being defended by William Waldegrave, its chief secretary, who as a former Chancellor of the Duchy of Lancaster — and as such responsible for the Office of Science and Technology — showed himself sympathetic to the need

for government support for basic science.

Peter Levene, a prominent industrialist who has been working for several years as the government's 'efficiency' expert, told the House of Commons select committee on science and technology last week that the pensions question was a 'highly complex issue', on which he had been asked to prepare a report for a cabinet committee.

But Levene refused to tell committee members of his conclusions, on the grounds that advice to ministers is confidential. Neither would he be drawn by claims from Labour party committee members that the government's determination to privatize the running of laboratories was based as much on ideology as practicality.

The Particle Physics and Astronomy Research Council is already known to be concerned about the legal and other costs of privatizing the management of the telescopes, which will have to be paid for out of its annual allocation. Although an official government statement said that these totalled £270,000 so far, informal estimates are that the final bill — given the need to brief US and Spanish lawyers concerning telescopes in Hawaii and the Canary Islands — could be as high as £2 million.

Others remain opposed on principle to the privatization of the running of the observatories. "The whole process has become unnecessarily cumbersome, and the best outcome would still be if it was abandoned," says Sir Martin Rees, the Astronomer Royal and Royal Society Research Professor at the University of Cambridge. **David Dickson**

BSE meeting reflects growing distrust of expert panels

London. The 'mad cow' crisis has exposed the need to reform Britain's system for providing scientific expertise to the government by making expert committees more representative and open, according to speakers at a meeting of politicians, public health officials, and farming and consumer organizations in London this week.

The meeting, entitled 'BSE: a sickness of government?', also heard proposals that the Ministry of Agriculture, Fisheries and Food (MAFF) should no longer have responsibility for animal welfare and its consequences for public health.

The meeting was organized by Charter 88, a body committed to constitutional reform. It appeared to confirm that the BSE crisis has increased public mistrust of the traditional idea that the safety of products can be adequately ensured by the existing system of expert committees. "Consumers have no confidence in the decision-making system that is supposed to protect us", said

Sheila McKechnie, director of the UK Consumers' Association.

Critics pointed out that, up to last December, the government's Spongiform Encephalopathy Advisory Committee (SEAC) did not include a single representative of the public health service. The meeting was told that there is a need to broaden the expertise of advisory committees, and in particular to include representatives of consumer organizations.

McKechnie says that the Consumers' Association has repeatedly asked MAFF for representation on SEAC and other advisory committees, but that its requests have always been refused on the grounds that it was not feasible, and that "we wouldn't understand the science". Consumer bodies can help "by asking the right questions" while gaining access to information.

Hugh Bayley, Labour member of Parliament for York, proposed that the minutes of all expert committees — except

those concerning national defence or similar restricted issues — should be made public, and that such minutes should record conflicting positions on issues.

Several speakers expressed concern that the media might create scares by handling such information irresponsibly. But the general mood of the meeting was one of criticism of what was described as the "closed" nature of scientific decision-making within government, and a feeling that greater public scrutiny was needed. The BSE crisis, Bayley said, had been a tale of "incompetence" by the government, and "impotence" on the part of parliament.

Much of the criticism at the meeting centred on the perceived conflict of interest in handling health issues within the MAFF. The Consumers' Association says that it is drawing up detailed proposals for a separate food agency that would be independent of both industrial and agricultural interests. **Declan Butler**

New Jersey outlaws genetic discrimination

Washington. The New Jersey state legislature last week gave near-unanimous approval to the most sweeping bill outlawing genetic discrimination yet passed in any of the 50 states.

But a controversial clause giving an individual property rights to genetic information was dropped after pressure from the biotechnology and pharmaceutical industries that are well represented in the state.

The bill is expected to be signed into law by New Jersey Governor Christine Todd Whitman (Republican) this week. It not only outlaws the use of genetic information to deny individuals jobs or health insurance, but also restricts how life and disability insurers may use such information.

In its revised form, the bill has received the backing of a wide range of interest groups, including the two industries involved, labour unions and the Roman Catholic church. The only obvious dissent has come from insurance groups, which testified against the bill last spring. They argue that insurers need all the information possible about applicants to accurately assess risk and avoid driving up rates on individual policies. They are said to have been "reluctantly coerced" into supporting the legislation.

Whitman had vetoed the bill in September, after both houses of the legislature had passed it unanimously (see *Nature* 383, 367; 1996). Her position reflected the concerns of the biotechnology and pharmaceutical industries, which had objected to a statement in the bill declaring genetic information to be an individual's private property.

The governor and the industries argued that this could have a 'chilling' effect on research, by exposing companies to law-suits for royalties by those whose DNA had been used to develop new products.

The property right declaration was subsequently removed from the bill. Supporters of the clause say that the political power of the pharmaceutical and biotechnology industries left them with little choice — but that the issue was a relatively minor concern when compared with the bill's broad anti-discrimination provisions.

Even in its modified form, the bill is "absolutely more far-reaching than any other", says Generosa Grana, a breast cancer specialist at Cooper Hospital in Camden, New Jersey, and an adviser to the New Jersey Cancer Commission, who helped draft the bill.

None of the advocacy groups fought

Whitman's demanded change "because there was so much [else] to lose", adds Karen Rothenberg, director of the Law and Health Care Program at the University of Maryland School of Law, and an expert on state genetic discrimination laws.

Not everyone agrees. George Annas, a lawyer and professor of public health at Boston University School of Public Health, says that "gutting" the property right clause has turned the bill into "an anti-genetic privacy act". He called it "bizarre" that "other people can own your genetic information, but you can't". And State senator Robert Martin (Republican), a law professor who was the lone Senate opponent of the revised bill, argues that Whitman's concern to protect industry may not have given enough protection to ordinary citizens.

The strength of the bill lies in its prohibition of discrimination not only on the basis of genetic tests, but of genetic information — a far broader term which includes family history, and can include individual history, physical examination and the results of other tests.

The bill is also broad in scope. It imposes restraints on life and disability insurers, in addition to employers and health insurers. Laws in other states have been narrower.

Under the bill, neither genetic information, nor an individual's refusal to submit to a genetic test or provide test results, can be used in decisions on hiring, firing and health insurance. Life and disability insurers may demand and use genetic information in underwriting, but must not use it 'unfairly'.

A life insurer, for example, could not use the fact that a woman is carrying a *BRCA1* mutation to decide whether to issue a policy, or what rate to charge, because this fact is no guarantee that she will develop cancer.

But the same insurer could legally refuse cover or charge higher premiums to somebody who carries the gene for Huntington's disease, as that person has a 100 per cent chance of developing the disease. In such a case, the insurer would have to base rates on actuarial data for Huntington's patients.

Only one state — Oregon — of the 12 others that have passed laws dealing with genetic discrimination includes a property right. An official now implementing the Oregon law says that the property right does not seem to have had any immediate impact. Michael Skeels, director of the state's Public Health Laboratory, adds that its implications for research will "take years" to become clear.

Earlier this year, the US Congress passed a law merely forbidding health insurers from using genetic information to discriminate against people who change or lose jobs. Pressure is growing for a broader federal law, and the issue may be addressed in the next legislative session. **Meredith Wadman**

Gene tests 'need research protocols'

Washington. An advisory committee to the National Institutes of Health (NIH) has recommended that genetic testing for breast and ovarian cancer be conducted only within strictly defined research protocols. This reverses an earlier position encouraging wider use of testing (see *Nature*, 380, 573; 1996).

Last week, the Advisory Committee on Research on Women's Health unanimously passed a resolution urging that genetic tests for breast and ovarian cancer be conducted only within "hypothesis-driven protocol studies" endorsed by NIH-approved institutional review bodies.

Typical studies, says the resolution, might address questions such as the positive predictive value of tests, and the appropriate medical management of those carrying mutations. The advice represents a refusal to endorse commercial genetic testing that does not incorporate hypothesis-driven research.

Last April the committee refrained from calling for testing to be confined to research protocols. One dissenter at the time was Linda Burhansstipanov, director of the Native American Cancer Research Program at the AMC Cancer Research Center in Denver, Colorado, who called the resolution "paternalistic". But last week she supported the revised

resolution, after the committee added a new, lengthy preamble. It includes a call for research on how poor, non-white and rural women can be guaranteed access to testing under research protocols.

Vivian W. Pinn, director of the NIH's Office of Research in Women's Health, says she agrees with the advisory committee. Access to genetics testing is important, but women "should know what it means", and such information is more likely to be both gathered and imparted in the research setting.

In adopting its position, the advisory committee joins the American Society of Human Genetics, the Advisory Council of the National Center for Human Genome Research, and the National Breast Cancer Coalition. In contrast, the American Society of Clinical Oncology has called for genetic testing to be made available outside research settings "as part of the preventive oncologic care of families".

The new recommendation comes two weeks after Myriad Genetics of Salt Lake City introduced commercial full-sequence testing of *BRCA1* and *BRCA2* genes, mutations which can confer a predisposition to breast and ovarian cancers. The company is charging \$2,400 for initial testing, and \$395 for tests of additional family members. **M.W.**

Germany keeps up the squeeze on CERN

Munich. The European Molecular Biology Laboratory (EMBL) in Heidelberg and the Institut Laue-Langevin in Grenoble, Europe's biggest neutron source facility, have been reprieved from a threatened cut in Germany's subscriptions. Last week the German parliament's finance committee approved a revised 1997 budget which backs away from the cut and is expected to be adopted shortly by the full parliament.

But there has been no such rethink of planned cuts in its subscriptions to CERN, the European Laboratory for Particle Physics, and two other European research centres.

The pressure has been increased by a further reduction of DM160 million (US\$106 million) in the budget of the ministry of research. As a result, Germany remains committed to reducing its CERN subscription by 8.5 per cent in 1997 and 1998, and by 9.3 per cent in the following two years. Unless such reductions are accepted by CERN's other member states, German officials are said to be prepared to give notice to quit the organization. It would then reapply for membership under different conditions.

At present, Germany is the largest contributor to CERN, whose budget consists of contributions from 19 member states according to a fixed formula, based on gross national product. Germany's determination to pay less means either that it must be given a special dispensation to reduce its subscription unilaterally, or that other members must reduce their contributions in line with Germany's cut.

At a CERN committee of council meeting earlier this month, three scenarios were put forward outlining how the threatened

German cut could be best handled to avoid disrupting the construction of the Large Hadron Collider (LHC).

A proposal to allow Germany special dispensation found little support. The scenario most likely to win the backing of CERN's council when it meets next month would involve reducing the subscription of all members, in line with Germany's planned reduction, with the resulting budget shortfall being made up by a mixture of internal savings and cash-flow adjustments.

Some member states may be tempted by the third, compromise scenario. This would introduce cuts in all contributions more gradually, starting with 6.5 per cent next year and reaching 8.5 per cent in 1999. But officials of the German research ministry are determined to resist this option.

Germany has been only slightly more flexible in its attitude towards cuts in its subscription to the European Synchrotron Radiation Facility in Grenoble, whose 12 member states pay contributions according to a formula determined in 1988 that depends on estimated use of the facility. Since July, Germany has reduced its proposed cut from 10 per cent to 7 per cent, in line with a proposal from Italy.

Even at this level, the cut will have a severe impact. But the facility has just had some good fortune in the form of a FF30-million (\$5.8-million) out-of-court settlement with a construction firm responsible for providing faulty flooring in an experimental area. This gives member states time to renegotiate their contributions, and absorb the German cuts.

Initially, the German government had

proposed cutting the subscription to EMBL next year by DM2 million (see *Nature* **382**, 285; 1996). But it has now backed off from this proposal, in line with its decision to protect and promote its underdeveloped biotechnology industry.

The Institut Laue-Langevin, which is financed by France, Germany and Britain, has also been let off the hook. A planned German cutback of 7 per cent will not be imposed. One reason seems to have been the fact that the institute was forced to cut back on staff and experimental instruments by 20 per cent when Britain reduced its contribution in 1993. In addition, Germany is backing a deal, due to be signed shortly, to reprocess the institute's highly radioactive waste at La Hague in France.

Christopher Llewellyn Smith, CERN's director-general, said on Monday that the laboratory faced "difficult times ahead", but that a decision by the committee of council to approve single-stage construction by 2005 — previous planning had been based on two stages, ending in 2008 — was "good news for CERN and for particle physics."

Alison Abbott & David Dickson

NIH retains research funds for the future

Washington. The US National Institutes of Health (NIH) has decided to celebrate a bumper funding year by holding back some of the money for spending in future years — raising alarm bells among biomedical research lobbyists who fear that such caution will deter Congress from increasing NIH funding in the future.

NIH officials say that a plan to issue about 300 multi-year grants, out of its total of 7,000 new grants to be issued in 1997, will provide stability for the young researchers eligible for the money. They argue that these researchers will not have to worry about any future cuts in the agency budget. But the strategy has the extra advantage, from NIH's point of view, that some of the 1997 money will thus pay for research done in 1998 or 1999.

In each year of the first Clinton administration, Congress has managed to get NIH to spend more money than the administration wanted; in 1997, for example, the administration asked for 4 per cent increase and the Congress granted almost 7 per cent — an extra \$300 million.

Tony Mazzachi of the American Association of Medical Colleges says that his organization has yet to decide its position on the funding plan, although he adds that "we are concerned about it". Other biomedical research lobbyists warned that the change could undermine the momentum for growth in the NIH budget.

Colin Macilwain

Decision deferred on modified maize

Paris. The European Commission last week postponed a decision on the approval of imports from the United States and elsewhere of Ciba Geigy's genetically-modified maize until the end of the year, citing the time needed for its three scientific committees — on food, animal nutrition and pesticides — to decide on the safety of the plant.

According to sources close to the commission, however, the pesticides committee has approved the maize, while the other two committees have already eliminated all "contentious issues" that could justify blocking its approval. Following the BSE crisis "the

commission wants at all costs to avoid to be seen to be rushing the science", admits one commission official.

The decision to delay coincides with demonstrations across Europe by environmentalist groups such as Greenpeace (above) against the import of genetically modified soya beans. □

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Vicente/Absolute

Germany urges universities to sharpen competition

Munich. The German government held high-level discussions this week with university heads and federal and *Länder* (state) politicians on how federal law could be changed to force universities to overcome their reputation for inefficient administration and lack of academic competitiveness.

The meeting, which was held with Jürgen Rüttgers, federal education and research minister, in Bonn, was called to discuss Rüttgers's radical hopes that Germany's overcrowded universities can be reorganized along the lines of the more competition-oriented British and US models, possibly as early as 1998.

Rüttgers wants to solve long-standing problems such as the uneven quality of teaching and research, and excessive study times. But to do so he needs the support of Germany's 16 *Länder*, which have responsibility for all cultural affairs — including education. The federal framework law on universities, known as the *Hochschulrahmengesetz* (HRG), merely gives general guidelines to the *Länder*.

Most *Länder* governments agree that there is a need to reform the HRG to ensure that universities start to move with the times. But they have reacted with hostility to Rüttgers's suggestions as to how this should be done. The *Länder* want the HRG to be restricted in scope, giving them more autonomy. But Rüttgers doubts the willingness of all *Länder* to introduce the changes he considers necessary. So he wants the HRG to be more prescriptive in some areas, and to relinquish some of its democratic principles.

The federal research ministry has drawn up a long list of proposed changes to the HRG to improve universities' efficiency. Many of the points meet with general agreement — for example the proposal that universities should have full control over their budgets and their choice of academic staff, without the involvement of the *Länder* governments.

But two of Rüttgers's suggestions are proving particularly controversial. First, in order to widen autonomy still further, he wants universities to have the right to choose their students, rather than being obliged to take all those who apply with appropriate school certificates. Second, he wants to abolish all permanent contracts for professors.

The *Länder*, as well as the universities

themselves, have mixed feelings about granting too much independence for universities. They fear that the changes, if implemented, could spell the end for the traditional concept of a 'German university', with its implicit recognition that degrees from all German universities are of equal value. They do not want to see Germany developing its own version of a Harvard or Cambridge university.

In any case, the prospect of being told what to do by the federal government touches a sensitive nerve. "It is unacceptable for the *Länder* to be told how to organize their universities," says Jürgen Zöllner, science minister of Rheinland-Pfalz.

But some of the more dynamic *Länder* have used their autonomy to introduce the type of changes that Rüttgers wants within the framework of the HRG. Bavaria, for example, recently introduced new rules to allow universities to offer professors short-term contracts in the interests of flexibility. The HRG does not forbid this. Hans Meyer, president of Berlin's Humboldt University, sees this as a "Bavarian crackpot idea". But Hans Zehetmair, Bavaria's science minister, stresses that the *Länder* should be allowed to make up their own minds on such issues.

Some *Länder* would also like to be able to introduce entrance examinations for potential students, but they are barred from doing so by a clause in the HRG. Other *Länder* defend Germany's long tradition of equality in education, a tradition that rejects even the mildest forms of elitism. Zöllner, for example, holds fast to his argument that it is "not that universities have the right to choose their students, but rather that they have a duty to educate young people".

The most likely outcome of the current attempts at reform is that the HRG will become more restricted in scope rather than broadened in the way Rüttgers would like. No-one wants the federal framework law to be abolished completely: both the *Länder* and universities accept that there is a need for general principles that each university has to follow.

Earlier attempts to reform the HRG have never succeeded in breaking through the strong barrier of the principles that divide federal and *Länder* governments. But the pressure of Germany's financial problems is weakening this barrier and, this time around, at least some reform is likely. Nevertheless, Rüttgers's hope that consensus can be achieved before the end of the year, and reforms put into effect by the end of next year, almost certainly underestimates the strength of his opponents.

Quirin Schiermeier

Canadian researchers hope for replacement for 'best' submersible

Ottawa. Canada's ocean scientists are waiting anxiously to learn whether the government will agree to provide a replacement for what is claimed to be the world's most effective robotic research submersible, which was lost at sea during a storm last month.

The Remotely Operated Platform for Ocean Science (ROPOS) broke away from a US research ship to which it was tethered in the Juan de Fuca Strait while being battered by 15-metre waves and 140-kilometre-an-hour winds.

Although ROPOS was insured, scientists are concerned that the insurance money may be used for purposes other than a replacement. Canada's federal fisheries and oceans department, which owned ROPOS, has already seen its research responsibilities reduced by budget cuts.

The submersible's insurance policy was arranged by a non-profit consortium called the Canadian Scientific Submersible Facility, formed this year to take over management of ROPOS. Initially the consortium was told by a fisheries official that the insurance money was to be used to buy other equipment. But at the end of last week the scientists learned that senior officials at the department were looking favourably at ROPOS's replacement, and a decision was expected shortly.

Kim Juniper, a biology professor at the University of Quebec at Montreal and a member of the consortium, says that the department had received more than 30 letters from the international oceanographic community supporting the replacement of ROPOS.

"If we don't replace ROPOS it will be absolutely devastating to the marine scientific community, not just in Canada but internationally," says Steve Scott, a geologist at the University of Toronto who is also a consortium member. "There would be a huge reduction in deep submergence capability in the world."

ROPOS is widely considered to be the world's best functional remotely-operated vehicle. The US vehicle Jason does not have such a successful track record. A French submersible, currently under construction, is likely to be expensive to operate, while the performance of a Japanese submersible is said to have been disappointing.

This summer ROPOS dived to almost 5,000 metres, and spent 17 hours on the seabed off the Aleutian Islands. According to Scott, failure to replace it would be a blow not only to the scientific community, but also to the government's ability to service the 25 per cent of its territory that lies underwater.

David Spurgeon



Zehetmair: striving for more flexibility.

Brookhaven feels the heat over reactors

Long Island, New York. Despite a reputation among scientists and regulatory authorities as a good environmental 'citizen', Brookhaven National Laboratory has become the target of an increasingly strident campaign by local activists to curtail its activities — if not to shut it down altogether.

The laboratory, which hosts the National Synchrotron Light Source and is now constructing the Relativistic Heavy Ion Collider, is situated on Long Island, the increasingly crowded 100-mile stretch of land east of New York city. Radioactive emissions from its two research reactors, which operate at 3 and 30 megawatts of thermal power respectively, are small, even by stringent US environmental standards.

But activists, spurred by recent reports of environmental contaminants in the area, have been trying to link these closely-monitored emissions to a high rate of breast cancer. Television news reports have gone further, linking the laboratory to the plight of individual children with cancer.

The argument over the Brookhaven reactors has its roots in Long Island's lengthy history of anti-nuclear protest, which culminated in the dismantling and removal of a newly-built nuclear power plant at Shoreham a decade ago. At the time, opponents argued that Long Island's 2 million inhabitants could not be evacuated safely in the event of meltdown at the power station.

Now many of the same activists are turning their attention to the laboratory. They have been placing advertisements in local newspapers asking parents to send in baby teeth for radioactive analysis. In exchange, they are offering free copies of a publication called *The Enemy Within: The High Cost of Living near Nuclear Reactors*. The critics are also calling for a 'consumer boycott' of the universities that set up Associated Universities Inc. (AUI), which runs Brookhaven for the Department of Energy (DoE).

Their immediate objective is to shut down the 30-megawatt High Flux Beam Reactor (HFBR) — an important neutron source for US scientists — as well as the smaller medical research reactor. "We want them to close down the reactors," says Bill Smith, a local activist, predicting that this will happen "in the next 12 months". Smith runs a group called Fish Unlimited, which believes that traces of radionuclides from the laboratory are harmful to marine life.

The protests are taking their toll on the Brookhaven laboratory. Managers are increasingly preoccupied with public relations, while staff scientists are taunted by their neighbours. "The toughest thing is being accused of jeopardizing the lives of children," says Karl Swyler, a physicist at the laboratory who now works on education

programmes inside Brookhaven and at local schools. The best thing the laboratory can do, he says, is "tell the truth and, when we screw up, admit it".

Brookhaven, which is used by scientists from a wide range of disciplines, has been trying to be more open about its activities, especially in the past two years. But the combination of this policy and hostility from its critics has served merely to draw more attention to the environmental problems.

For example, when the local Suffolk County health department found trichloroethane in groundwater near Brookhaven last year, DoE quickly agreed to pay for



Campaigners link physics lab (above) to cancer in children and want teeth for analysis (below right).

public water supplies for homes with wells in the affected area — even though Brookhaven scientists believe the contamination did not come from the laboratory. Some residents are now suing the laboratory for \$1 billion, claiming that the DoE's action implies guilt.

More recently, critics have been focusing on the laboratory's release of radioactive tritium from the HFBR into the Peconic river, which flows into Peconic Bay in Long Island's East End. They charge that the level of tritium in this river is 100 times that of any other in New York State, and have not been placated by the laboratory pointing out that the concentration is still only 10 per cent of the US drinking water standard.

In its effort to win back support, Brookhaven is sending its scientists frequently into local communities to explain their work. AUI has also provided facilities to support moderate community groups concerned about the lab.

In some cases this policy seems to be working. Jean Mannhaupt, a housewife and long-time critic of the lab, who asked for and got help from AUI to set up an 'advocates' office' at Brookhaven, says the laboratory's approach has been transformed for the better. She does not want the reactors closed, but is pressing for quicker action to remove spent fuel from temporary storage at the lab.

At a meeting two weeks ago at Southampton, a wealthy and environmentally sensitive community 20 miles east of the laboratory, senior Brookhaven managers sought to provide reassurance that the laboratory produces benefits for Long Island, and that past and present emissions are too small to threaten human health.

Bob Casey, head of safety and environ-

mental protection at the laboratory, explained to the meeting, attended by about 100 people, the meaning of a curie of radiation, or a rem of exposure. To dramatize radiation risks, critics of the laboratory often talk about "picocuries"; Casey explained that there were 25 billion picocuries of tritium in a typical wristwatch.

According to Casey, the maximum additional exposure of someone living next to the laboratory will be 1 millirem, compared to a typical ambient exposure on Long Island of 200 mrem. Sue Davis, associate director of the laboratory, tried to quash a widespread perception that it performs nuclear weapons research. Only 2 per cent of its research work is defence-related, the laboratory says, and that is aimed at halting nuclear proliferation.

Public response to such assurances remains split. One activist, Roger Snyder, who has been picketing the laboratory gates every Saturday for a year, summed up the position of the critics. "What you are saying is 'trust the experts'. Well, we trusted you for 30 years," he told the Brookhaven officials. "I'd say your record is pretty poor."

Such attacks seem likely to continue. They are supported by a small but hardy group that includes Helen Caldicott, a physician and veteran anti-nuclear campaigner, and Jay Gould, a statistician and prominent critic of the lab, whose group, the Radiation

BABY TEETH WANTED!

Grants from the Methodist Church and East End families, concerned about high levels of radioactivity recently found in the Peconic River, will enable the non-profit Radiation and Public Health Project (RPHP) to undertake a two year study of the health effects of radioactivity levels in Suffolk County. The study will measure the strontium-90 content and other long-lived chemical pollutants levels found in baby teeth from all sections of Suffolk County. Deer teeth and fish skeletons from the Peconic River will also be examined as part of the study.

For more information about where to send the baby teeth please call Fish Unlimited at 749-3474 or contact them by Fax at 749-3476.

Those sending a baby tooth will be informed of the results in confidence, and will be sent a free copy of the recent RPHP publication *The Enemy Within: The High Cost of Living near Nuclear Reactors*, otherwise available for \$17.50 by calling 1-800-626-4848.

and Public Health Project, was responsible for the advertisement asking for baby teeth.

At the time of the dispute over the Shoreham power station, some scientists at Brookhaven expressed their personal support for the proposed reactor. Now there is clearly little love lost between this group and the activists who have focused their attack on the laboratory.

But so far the opponents have failed to mobilize widespread public support, and some feel that the laboratory has become paranoid about the activities of a relatively small group of citizens. Peter Boody, editor of the weekly *Southampton Press*, says that opposition to Brookhaven does not run very deep. But he says that the laboratory is "quite obsessed" by it. "They feel like a great whale with harpoons hanging out of its body."

Colin Macilwain

Board lifts its threat to tenure at University of Minnesota

Washington. The board of regents of the University of Minnesota has withdrawn a controversial proposal that faculty members had feared would have undermined security of tenure for the 3,000 tenured academics at the university.

Faculty members at the university had vigorously opposed changes in the tenure rules proposed by the board in September, and threatened to form a trade union. The proposals would have established new sets of circumstances — such as the abolition of programmes — under which tenured staff could lose their jobs.

The board has now said that it will impose the changes at the small law school, which has voted not to form a union. But Tom Reagan, chair of the board, said that, for the rest of the university, “we will not be revisiting the tenure code for at least a year and a half, and probably never”. □

Meteorite meeting in Japan

Washington. Japanese scientists are to meet tomorrow (22 November) at the National Institute of Polar Research in Tokyo to consider how the nation's large Antarctic meteorite collection can be used to help answer the question of past life on Mars. Japanese science officials promised to cooperate with the United States in such research when they met President Bill Clinton in August at the time of the announcement about life on Mars by the National Aeronautics and Space Administration. But no strategy has yet been formulated, says Akira Shimoyama of the University of Tsukuba.

Japan's polar institute houses some 9,000 Antarctic meteorites — more than exist in the US collection. Although only two are known to come from Mars, nearly half the collection has yet to be classified,

according to Shimoyama, due to limited manpower. Japanese collectors are expected to gather another 2,000 to 3,000 meteorites from Antarctica next year, he says. The symposium will address current scientific knowledge of the existing collection and of martian meteorites in general, as well as how Japan's two martian rocks should be studied. □

Europe reviews CJD surveillance

London. The European Commission is planning to hold a meeting next month in a bid to thrash out a common system for reporting cases of Creutzfeldt-Jakob disease (CJD). The move follows the completion of a survey by the commission that reveals shortcomings in CJD surveillance networks in the European Union.

The survey shows that comparison of CJD data throughout Europe has been made “difficult” by the many different methods used by member states to diagnose and report cases (moreover, while 11 countries had surveillance systems at the time of the survey, only five had in 1990). The survey concludes that these produce data that are “too disparate to allow an overall analysis of the cases”, and that it is “urgent” to introduce a more systematic approach. The meeting will include heads of national CJD surveillance networks and health officials. □

Pakistan's science minister

London. Pakistan has appointed a new science minister, for the second time in three months. Abida Hussain, a former diplomat, takes over from Nawaz Khokhar, who was sacked earlier this month by President Farooq Leghari, along with the rest of the government of prime minister Benazir Bhutto, on charges of corruption. Khokhar was arrested by police last week and charged with fraud.

The science minister's post remained vacant for three years before Khokhar's surprise appointment in a cabinet reshuffle earlier

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this year. Bhutto is widely believed to have rewarded him with the ministerial post when he left the main opposition Muslim League to join her ruling Pakistan People's Party. His successor, Hussain, Pakistan's former ambassador to Washington, may have to relinquish the job herself in less than three months. Elections are scheduled for next February. □

Israel sets up science committee

Jerusalem. A parliamentary committee on science and technology has been set up to assist the Knesset, Israel's parliament, to address an area that the legislature has so far neglected. The creation of the committee was announced by Dan Tichon, speaker of the Knesset, last week. Tichon told the presidents of Israel's universities that science and technology are national priorities, and that many other countries' parliaments already have committees to oversee these issues. The decision, he said, was made at the recommendation of Ze'ev Binyamin Begin, minister of science. □

Challenge over 'tobacco' sacking

London. A senior official of the Medical Research Council (MRC) who was dismissed last week for publicly criticizing a decision to accept a tobacco industry grant for a study on the effects of nicotine on Alzheimer's disease, is to challenge the decision at an industrial tribunal.

An MRC statement said the decision to dismiss Mary Rice, the council's former head of public communication, for "gross misconduct" had been taken with considerable regret. The statement added that Rice could not criticize the council in public while an employee. But Rice says she has "no regrets" about voicing her criticisms, which, she says, "were in the MRC's best interests as a whole". The £147,000 (US\$222,000) donation was given by BAT Industries to the MRC's Neurochemical Pathology Unit in Newcastle. □

Ebola source identified

London. A doctor in Gabon has been identified as the source of a new outbreak of the deadly Ebola virus in South Africa. The doctor has now recovered, but had already infected a woman who flew to a Johannesburg clinic for treatment last month. This woman in turn infected a nurse at the clinic, who is now in a critical condition.

Health officials are trying to trace all patients who may have come into contact with the nurse in the past three weeks. She worked at the Morningside Clinic in Sandton, one of Johannesburg's affluent white suburbs. Officials are worried that the virus may spread overseas if one of her patients has left the country. □

'Science ambassadors' return

Paris. Twenty postdoctoral or postgraduate researchers from Europe's big science facilities will return to their old schools in 16 European countries later this month to tell pupils about their centres' work. The scheme is being organized as part of the European Union's "European Week for Scientific and Technological Culture".

The 20 'science ambassadors' will represent facilities such as the European Laboratory for Particle Physics (CERN), the European Molecular Biology Laboratory (EMBL), the European Space Agency (ESA) and the European Synchrotron Radiation Facility (ESRF), as well as the Joint European Torus (JET). □

Gold medal for flower analysis

Munich. Enrico Coen of the John Innes Research Centre in Britain has won this year's gold medal of the European Molecular Biology Organization for his work on the molecular and genetic analysis of flower development. The medal, together with a prize of DM15,000 (US\$10,000), was presented at the organization's meeting on 'Frontiers of Molecular Biology' in Rome earlier this month. □

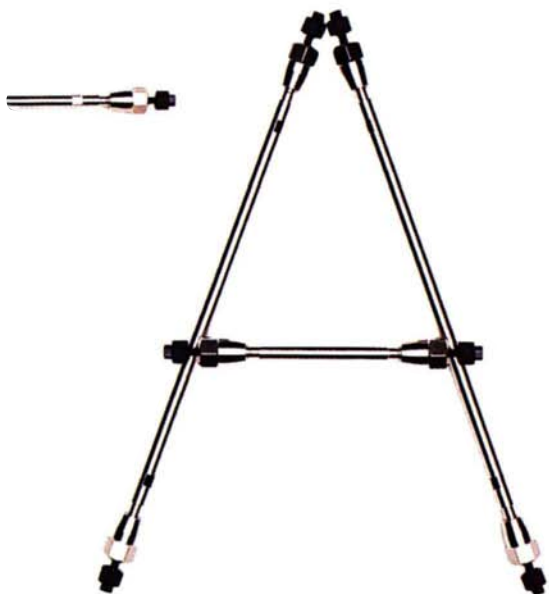
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Don't forget the Universe

SIR — In June of this year the scientific programme committee of the European Space Agency (ESA) approved the COBRAS-SAMBA satellite as the agency's third medium-size mission, and the first devoted to the critical problems of cosmology. COBRAS/SAMBA will map the whole sky with unprecedented precision over the wavelength range from 3 to 0.1 millimetres. After the extraordinary success of NASA's satellite COBE, we now know that the design specification of COBRAS/SAMBA should allow all the observationally accessible structure of the Universe at an age of only 300,000 years to be mapped in detail. Such maps are expected to yield precise measures of the age, matter content and curvature of our Universe, as well as determining whether a cosmological constant in Einstein's equations has affected cosmic evolution. They will demonstrate whether our current understanding of the history of the Universe is correct. Such a demonstration would clearly have profound philosophical implications going well beyond technical cosmology and comparable, perhaps, to Magellan's demonstration that the Earth is round. Such a crucial addition to our knowledge of the world can occur only once, and COBRAS/SAMBA has the chance to make this step.

Unfortunately, the Ariane 5 disaster, occurring just before the ESA committee meetings, has caused serious planning problems. The scientific programme committee, while approving COBRAS/SAMBA, decided that final confirmation must await assessment of the effects of the loss of Cluster, a mission to study the structure of the Earth's magnetosphere which was destroyed with Ariane 5. Your 14 November issue reported that ESA's external space science advisory committee recommended that a replacement for Cluster be launched in 1999, and that the cost of ECU210 million be found by delaying the Far Infrared Space Telescope by six months and the fourth medium mission (expected to be a planetary mission) by two years (*Nature* 384, 99). We are writing to emphasize that the costs of the Cluster replacement should not delay the whole of ESA's long-range Horizon 2000 plan. In particular it is critical that COBRAS/SAMBA be launched at or before the current outline date of 2004. (It was to be 2003 before a cost-saving exercise carried out by ESA earlier this year).

COBE generated worldwide interest in using the microwave background to understand the origin of our Universe. Cosmology has now become highly competitive and is supported strongly in many countries. There are, worldwide, rapid developments in microwave technology, and in experiments on the ground, in balloons, and in simpler

satellites. Any delay could jeopardize COBRAS/SAMBA's status as the definitive mission; a unique opportunity for European science would then have been lost.

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First for biotech

SIR — I was pleased to learn that the British government is to appoint a Human Genetics Commission to regulate the complex field of genetic diagnosis and technologies, in addition to the Advisory Committee on Genetic Testing (see *Nature* 381, 640; 1996). Although you stated that the latter committee was thought to be the "first of its kind", with responsibility for ensuring that genetic screening tests are "supplied safely and used ethically" (*Nature* 379, 195; 1996), for more than a year the Norwegian National Board of Health has had a Medical Biotechnology Advisory Board with these tasks as well as broader responsibilities like those of the Human Genetics Commission.

The Norwegian regulations took effect on 1 September 1994, and cover human reproductive technology, research on embryos, all kinds of genetic testing on embryos, fetuses and born people, as well as gene therapy. Although it is important that regulations must not make valuable clinical practice too difficult, it seems clear that this field should not be left open to market forces. We are convinced that such a law is necessary, and that regulation will prevent many of the difficulties reported from other countries (see *Nature* 378, 120; 1995).

The borderline between genetic variation and disease cannot be conveniently defined in legal terms, and in decisions on how to regulate this field there is no substitute for the detailed clinical and scientific knowledge provided by a good advisory board. Summing up our first year's experience, the advisory group has been indispensable, and we are convinced that the British authorities will not regret the decision to appoint their group.

Ola Myklebost

(Secretary)

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Police yourselves

SIR — It was not my intention in my report on "Fringe science in the 104th Congress", discussed in your leading article "Don't ban sceptics" (*Nature* 383, 745; 1996), to imply that dissenting scientific viewpoints should be suppressed. Nor was it my intention to reflect adversely on the character, integrity, credibility or qualifications of the "sceptic" scientists who testified before our committee. My point was rather that scientific disagreements are better resolved within the scientific community than by Congress.

Members of Congress are not particularly well qualified to make scientific judgements or to conduct peer review of scientific assessments. Our best assurance of objective, high-quality science remains the peer-review system, as imperfect as it may be. In that regard, I encourage those who doubt the so-called 'scientific consensus' to spend less time spinning their views to the media and more time convincing scientific colleagues of the merit of those views through time-tested scientific methods such as proposing and testing alternative hypotheses.

To be sure, Congress does have a role in ensuring that the scientific process is truly open to legitimate alternative viewpoints. The report also points out that scientific certainty is one among many legitimate policy issues that Congress must consider in making regulatory policies. In that light, Congress should always be open to hearing a variety of policy perspectives of those with experience and expertise in the science and policy arena.

George E. Brown Jr

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Next step in space

SIR — Your item on the space science workshop being hosted by the National Research Council (NRC) in advance of the forthcoming 'space summit' may convey a misleading impression (*Nature* 383, 372; 1996).

The expert group convened at the request of the National Aeronautics and Space Administration (NASA) will carry out a technical assessment of current NASA programmes in light of previous NRC recommendations, and prepare a discussion paper outlining "appropriate next steps" in broad terms. The group is not charged with setting mission or programme priorities or developing a budget analysis or recommendations. The paper will not be a formal NRC report but a summary of the workshop proceedings.

Marc S. Allen

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Focus on butterfly eyespot development

H. F. Nijhout

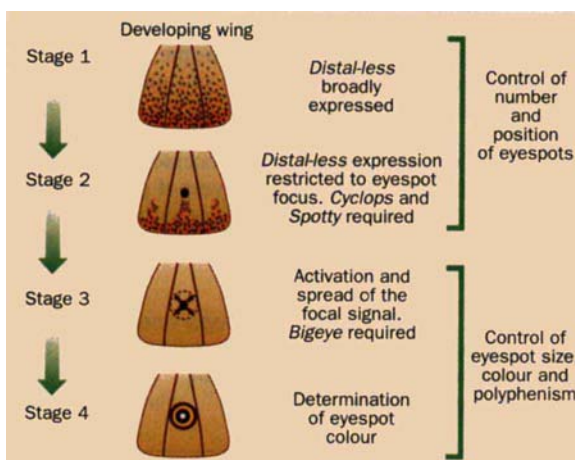
The beautiful eyespot patterns on the wings of butterflies help them to evade predators, but they also present biologists with a unique opportunity to investigate the reciprocal interactions of development and evolution.

THE colour patterns of butterflies have evolved as devices for visual communication — they serve both as sexual signals in courtship and as camouflage. They are also used by distasteful species to warn predators, and by palatable species to mimic the distasteful ones. The most conspicuous elements in the colour patterns of many butterflies are the eyespots, which are sets of concentric rings of contrasting colours that are believed to mimic the eyes of a large animal. On page 236, Brakefield *et al.*¹ explore the genetic, developmental and evolutionary mechanisms that underlie the ontogeny (growth and development) and diversity of eyespot patterns. This paper is the product of a unique collaboration between molecular geneticists, developmental biologists and evolutionary biologists, and it opens the door to a new stage in the integration of developmental and evolutionary biology.

Eyespots presumably evolved as a defence mechanism because they can startle and confuse potential predators^{2,3}. So eyespot patterns are interesting from an evolutionary point of view, as they represent a visually compelling product of natural selection. Eyespots are also attractive from a developmental point of view because they are structurally simple. The wing surface on which they develop is a monolayer of cells composed of alternating parallel rows of epidermal cells and scale cells. Typically, each scale cell synthesizes a single pigment, and the overall colour pattern is built up as a finely tiled mosaic of scales that bear different pigments.

Perhaps the most fascinating thing about the butterfly colour pattern is that it is made up of a number of semi-independent pattern elements. This 'modu-

lar' construction is made possible by the fact that eyespots and other pattern elements develop near to discrete signalling sources that organize the pattern of pigment synthesis in their vicinity⁴. They act in much the same way as embryonic inducers, by emitting biochemical signals that alter the developmental programme of the surrounding cells.



A four-stage model for butterfly eyespot development as proposed by Brakefield *et al.*¹.

Because the signals from these organizing centres do not seem to spread very far, pattern formation on the wing is essentially a local process.

Pattern development in one region of the wing might, therefore, be uncoupled from that in other regions. If this is correct, it implies that one portion of the pattern may be able to respond to natural selection without altering other nearby portions of the wing pattern in ways that might be unfavourable to the animal. So eyespot patterns present us with an opportunity to investigate the reciprocal interactions of development and evolution. To date, research in this field has focused largely on the identification of developmental mechanisms that might constrain variation; on the patterns of genetic correlation among characters; and on the identification of evolutionarily primitive developmental mechanisms (such as homeobox genes) among distant taxa. Much less work has been done on forming an integrated view of how developmental and genetic mechanisms interact with natural selection to allow an animal to adapt to its environment.

Brakefield *et al.*¹ address three aspects of the development and evolution of eyespots: the positional specification of their organizing centres (the foci); the control of eyespot size around a focus; and the way in which the eyespot and the developmental system that generates it have been altered by selection — past and present. Using a combination of molecular probes for regulatory genes,

a trio of newly discovered genes that affect eyespot development, and classical methods in embryology and evolutionary biology, they identify four stages in the developmental pathway of an eyespot (see figure).

The process begins late in larval life, in the wing imaginal disc, with the expression of the regulatory gene *Distal-less* (*Dll*)⁴. The *Dll* gene has been cloned⁵, and in the first stage of eyespot development it is broadly expressed. In the second stage, expression is gradually restricted to a single rounded spot that will become the focus of an eyespot. The spatial progression closely resembles that predicted by a lateral-inhibition model for

origin and placement of foci on butterfly wings⁶. Brakefield *et al.*¹ have used mutational analysis to identify two hitherto unknown genes, *Cyclops* and *Spotty*, which seem to be part of the process that determines the number of foci and places them in their precise locations on the wing. The third stage is the activation (during the early days of the pupa) of the focal signal that will induce the eyespot. The size of the eyespot that is produced is regulated in part by the third new gene, *Bigeye*. The final stage is determination of the colour of the eyespot. This is not a property of the focal signal but of the cells that receive the signal, as shown by the fact that when foci are transplanted to different locations on the wing, they induce eyespots of different colours.

A twist that adds an additional feature of interest to this system is that the colour pattern of one of the species studied, *Bicyclus anynana*, exhibits what is called a 'seasonal polyphenism', a form of phenotypic plasticity. Polyphenism is the ability of individuals with identical genotypes to develop two or more distinct alternative phenotypes in response to certain en-

1. Brakefield, P. M. *et al.* *Nature* **384**, 236–242 (1996).
2. Blest, A. D. *Behaviour* **11**, 209–256 (1957).
3. Wickler, W. *Mimicry* (McGraw-Hill, New York, 1968).
4. Nijhout, H. F. *The Development and Evolution of Butterfly Wing Patterns* (Smithsonian Inst. Press, Washington DC, 1991).
5. Carroll, S. B. *et al.* *Science* **265**, 109–115 (1994).
6. Nijhout, H. F. *Science* **265**, 44–45 (1994).
7. Shapiro, A. M. *Evol. Biol.* **9**, 259–333 (1976).
8. Moran, N. A. *Am. Nat.* **139**, 971–989 (1992).
9. Nijhout, H. F. *Insect Hormones* (Princeton Univ. Press, 1994).



Watch out! The distinctive eyespots on the wings of many butterflies are thought to mimic the eyes of a large animal.

environmental stimuli^{1,7-9}. When *B. anynana* larvae are reared at warm temperatures, the adults develop rather large and conspicuous eyespots and a highly contrasting colour pattern that is typical of butterflies that fly during the wet season. But if the larvae are reared at cool temperatures, the eyespots do not develop much, if at all, and the overall colour pattern is darker, with little contrast, and typical of that found in animals that fly during the dry season. The effect of temperature is clearly indirect because, as the authors show, it is possible to select for strains that express either the wet-season form or the dry-season form in the laboratory (that is, in the absence of

environmental influences). Other insect polyphenisms are based on hormone-induced switches in developmental pathways⁷⁻⁹, so artificial selection may well have affected some component of this endocrine mechanism.

The evolution of eyespots comes about through changes in the four-stage developmental sequence. Brakefield *et al.*¹ have shown that species that differ in the number of eyespots they possess diverge in the early stages of eyespot determination. Phenotypic plasticity of the eyespot, by contrast, occurs through changes in the later stages of the process. The exact points at which development diverges in these two extreme cases of eyespot evolution is as yet unclear. But the new tools developed by Brakefield *et al.* should now allow us to begin a detailed analysis of the processes that are affected by the various eyespot genes, and to acquire a deeper understanding of how the developmental process itself evolves. □

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PROTEIN TRAFFICKING

Transportin nuclear proteins

Colin Dingwall

THE constant exchange of macromolecules between the nucleus and the cytoplasm occurs through the nuclear pore complex and represents a major investment of energy by the cell. In addition, these transport processes are regulated by mechanisms in which molecules are restricted to a particular compartment until specific signals stimulate their migration. It is generally thought that there is one major pathway of nuclear protein import, which is mediated by nuclear localization signal (NLS) sequences that are characterized by one or more clusters of basic amino acids¹. Proteins lacking an obvious 'basic-type' NLS are thought to piggy-back into the nucleus in association with nuclear proteins that do. But studies described in *Cell*² and *Science*³ have identified a new receptor that mediates nuclear protein import by a route that is distinct from the basic-type NLS pathway.

The focus of the study by Pollard *et al.*² is mammalian hnRNPA₁, one of a set of approximately 20 proteins that are able to

form complexes with heterogeneous nuclear RNA (hnRNA), and are involved in many aspects of RNA metabolism, including RNA processing and transport⁴. These proteins (designated hnRNPA to hnRNPU) are found at high concentrations in the nucleus, and proteins of one subgroup (hnRNPC₁, hnRNPC₂ and hnRNPU) have the basic-type NLS. However, members of a different subgroup — which includes hnRNPA₁ — do not. Yet all of these proteins constantly 'shuttle' between the nucleus and the cytoplasm, and both the entry and export steps show characteristics of being signal-mediated processes: they are saturable and temperature-dependent^{5,6}.

In hnRNPA₁ there is a single stretch of about 38 amino acids, designated M9, that is both necessary and sufficient for nuclear import and export; and related sequences are present in other members of the subgroup to which hnRNPA₁ belongs^{5,6}. The M9 sequence is unlike either the basic-type NLS or the nuclear-export signal that

has been identified in HIV Rev and the protein kinase A inhibitor peptide⁷. But the M9-mediated import step has similar biochemical characteristics to the basic-type NLS protein-import pathway — it is inhibited by wheat-germ agglutinin and by ATP depletion. However, M9-mediated import cannot be competed out by saturating amounts of basic-type NLS peptide², indicating that it acts through a specific, hitherto unknown receptor. Incubation of a cell extract with an M9 affinity matrix produces a depleted extract that can still support the nuclear import of a protein with a basic-type NLS, but not a protein with the M9 NLS. Using the yeast two-hybrid system, Pollard *et al.*² have identified a human protein called transportin that interacts specifically with the M9 sequence, and they have shown that the recombinant protein can promote the import of an M9 fusion protein *in vitro*.

Aitchison *et al.*³ used a different approach to identify a protein in *Saccharomyces cerevisiae*, Kap104p, which can be isolated as a complex with two nuclear messenger RNA-binding proteins that are similar to the vertebrate shuttling hnRNPs. So Kap104p seems to be the functional analogue of transportin³. Interestingly, both the transportin and Kap104p sequences show homology to one of the components of the receptor for the basic-type NLS (ref. 8). This receptor consists of two subunits (relative molecular masses 60K and 97K) known as importins, or karyopherins- α and - β , respectively. *Xenopus* importin- α , and one human homologue, have been shown to bind directly to basic-type NLSs. Importin- β docks the receptor complex at the nuclear pore complex, and it is to this subunit that transportin shows significant homology. So during the import of hnRNP, the requirement for an importin- α -like function is bypassed and the M9 nuclear localization sequence interacts directly with its receptor protein which, by analogy with importin- β , may dock directly at the nuclear pore complex. Consistent with this, the binding studies of Aitchison *et al.*³ show that Kap104p can interact directly with a set of nuclear pore proteins that is similar to the set that binds to the β -subunit of the basic-type NLS receptor.

The shuttling hnRNP proteins are thought to be directly involved in the export of mRNA from the nucleus, and the hnRNPA₁- and hnRNPA₂-type proteins are stably associated with RNA during the export event². Point mutations in the M9 sequence of hnRNPA₁ abolish both the import and export of the protein, indicating that M9 is involved in both processes, although there is some evidence for an export signal elsewhere in the transported protein⁹. If the M9 sequence is directly involved in export,

does transportin mediate this step too? In the case of the yeast protein, a temperature-sensitive mutant of Kap104p was isolated. At the non-permissive temperature, levels of Kap104p declined rapidly in the cell and the mRNA-binding protein Nab2p was redistributed to the cytoplasm. But only a weak reduction in mRNA export was observed, indicating that Kap104p is primarily involved in nuclear import, and not mRNA export.

Although the nuclear-import pathway that is mediated by transportin/Kap104p is clearly distinct from the basic-type NLS pathway, the nuclear GTPase Ran is likely to be a common factor in the two pathways. Ran is a soluble factor that is essential for nuclear protein import (basic-type NLS), and the nuclear GTPase cycle is crucial for the correct execution of a large number of nuclear functions including protein import and RNA export. Ran is also required for the import of small nuclear RNPs (snRNPs) into the nucleus. Like hnRNP import, this process is mediated by a nuclear localization signal and receptor that are distinct from the basic-type NLS¹⁰.

It is clear that both transportin and Kap104p mediate a distinct nuclear import process, but the current evidence suggests a difference in their roles in RNA export. The M9 sequence, and so by implication transportin, functions in export from the nucleus in higher eukaryotes⁶, but Kap104p appears to be involved in import only³.

Whatever the final outcome, a rough calculation illustrates the significant contribution of this pathway to the transport of proteins through the nuclear pore complex. The total number of molecules of hnRNPA₁ and hnRNPA₂ in each HeLa cell nucleus is estimated to be $70\text{--}90 \times 10^6$. At mitosis, these proteins are released into the cytoplasm and re-imported after the nuclear membrane has re-formed. If re-accumulation takes one hour, then the import rate during this period is around 500 molecules per minute per pore complex. □

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Cool gaze at heartless galaxies

Gerry Gilmore

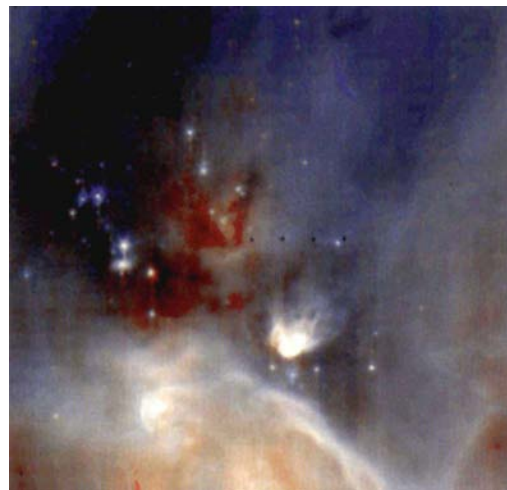
STAR formation is ubiquitous, but almost entirely mysterious. Some galaxies completed their star formation long ago, whereas many form stars sporadically, punctuated by long, pregnant pauses. Most spiral galaxies, like the Milky Way, seem to have formed stars at a fairly constant, low rate for the whole lifetime of their disk. Other galaxies again, especially those in the throes of major mergers, are forming stars so rapidly that they will exhaust their gas reservoirs very soon. How do stars form, and why do they form at such different rates? Are the most luminous galaxies powered by extreme rates of star formation, or do they harbour black-hole-powered active nuclei as well? A clear answer to this last question, and partial answers to the others, are found in a special issue of *Astronomy and Astrophysics* this month¹⁻⁴, which reports the first scientific results from the Infrared Space Observatory (ISO)*.

ISO is the first infrared astronomy satellite to have high spatial and spectral resolution, good sensitivity and wide wavelength range. Its 60-cm telescope supplies light to four instruments — two spectrographs, a long-wavelength photometer and a camera operating at wavelengths of 2 to 20 micrometres — all cooled to two kelvin by 2,500 litres of superfluid helium. Targets range from the Solar System to the most distant proto-galaxies, but perhaps the most substantial advances reported to date are in two related fields: star formation in our Galaxy, and the nature of the most luminous known galaxies, in which extreme rates of star formation are perhaps complemented by massive black holes in active nuclei.

The best models of these luminous and ultra-luminous infrared galaxies have treated them as mergers of two large, gas-rich galaxies, with very rapid star formation triggered by the interaction of the huge gas masses, and with an active galactic nucleus (AGN) fed by gas flow into the central regions but hidden by dust (for a review see ref. 5). The most infrared-luminous galaxies in particular seemed not to be forming many of the most massive stars, suggesting both the presence of an AGN to provide the enormous infrared power, and that the mass distribution of stars formed in a starburst is different from that produced

under more sedate circumstances.

Starbursts have softer radiation spectra than AGN, and so they excite different levels of ionization in the galactic gas which result in different emission-line ratios. It is these ratios that we use to discriminate between the two types of source, in effect determining the temperature of the radiation field. At optical and near-infrared wavelengths, however, very large corrections must be made for



Newborn stars, still swaddled by dust, are revealed in the infrared by ISO. These are in Rho Ophiuchi, about 500 light-years away in our own Galaxy, but the process may be almost identical to the starbursts that power some of the brightest distant galaxies. (ESA/ISO, CEA Saclay and ISOCAM Consortium\Image processed at IAS Orsay.)

colour-dependent absorption by dust: a typical model requires a factor of 10^4 reduction in short-wavelength intensity, which introduces the possibility of systematic uncertainties. But ISO can see directly the mid- and far-infrared emission lines, which suffer very little absorption and are sensitive to a very wide range of densities and ionization levels.

Already, ISO has been used to study three ultra-luminous infrared galaxies, and a sample of less extreme star-forming galaxies¹. The remarkable result is that they do not harbour AGN — they are powered solely by rapid star formation, and the stars form with the same mass distribution as that seen in the Milky Way. In one lovely example, of two intersecting disk galaxies known as the Antennae, it is even possible to resolve the spot where the two disks currently cross, and to see the progression of star formation across the disk as the two galaxies orbit through each other².

At the other end of the distance scale, in regions of star formation in our Galaxy, one sees a lot of detail: dark, cold cores; bright, ionized filaments; newly formed

1. Dingwall, C. & Laskey, R. A. *Trends Biochem. Sci.* **16**, 478–481 (1991).
2. Pollard, V. W. et al. *Cell* **86**, 985–994 (1996).
3. Aitchison, J. D., Blobel, G. & Rout, M. P. *Science* **274**, 624–627 (1996).
4. Dreyfuss, G. et al. *Annu. Rev. Biochem.* **62**, 289–321 (1993).
5. Siomi, H. & Dreyfuss, G. *J. Cell Biol.* **129**, 551–560 (1995).
6. Michael, M. W., Choi, M. & Dreyfuss, G. *Cell* **83**, 415–422 (1995).
7. Gerace, L. *Cell* **82**, 341–344 (1995).
8. Görlich, D. & Mattaj, J. *Science* **271**, 1513–1518 (1996).
9. Weighardt, F., Biamonti, G. & Riva, S. *J. Cell Sci.* **108**, 545–555 (1995).
10. Palacios, I. et al. *J. Cell Biol.* **133**, 485–494 (1996).

*ISO is a collaboration between the European Space Agency, ISAS of Japan and NASA.

stars busily heating, evaporating and illuminating the region; and complex molecules cooling the region, being created and destroyed in the process. To understand star formation, one must understand the relationships between all these phenomena. One long-suspected chemical change in star-forming regions is the formation of hot water—long suspected, but not observed (except as a microwave maser line in special circumstances) because water in the Earth's atmosphere blocks the important wavelengths. But ISO, being above the atmosphere, has already seen hot water, cold water, ice, carbon monoxide, carbon dioxide, polycyclic aromatic hydrocarbons, silicate, methane... and so the list goes on. The study of the molecular environment of star-forming cores is set to become an observational science *par excellence*³.

A critical issue in understanding star formation is the density structure of the cold, dense cores of molecular clouds, which are believed to be the precursors of star-forming regions. The low temperatures of these clouds and their extremely high optical obscuration have hampered observations, because we have been limited by the low spatial resolution of ground-based, sub-millimetre telescopes, and by the restriction to densities and temperatures probed by molecular transitions that happen to be observable through the Earth's atmosphere. One of the nearest star-forming regions—rho Ophiuchi, some 160 parsecs away—has now been analysed by ISO. Dense cores are indeed seen, the distribution of ionizing radiation can be mapped out, and detailed studies have begun of the evolution of a region in which new hot stars compete with far-infrared cooling and complex chemical and molecular evolution to stimulate or prevent further star formation⁴.

In combination, these first detailed and unobscured studies of nearby and distant star formation are a firm empirical basis to build on. The early ISO results are extremely encouraging in showing that star formation differs more in rate than in kind; that reliable dust-absorption-free data can be obtained; and that the Universe is kind, at least sometimes, in providing clean examples of star-forming galaxies uncontaminated by AGN. □

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Three paths to stress relief

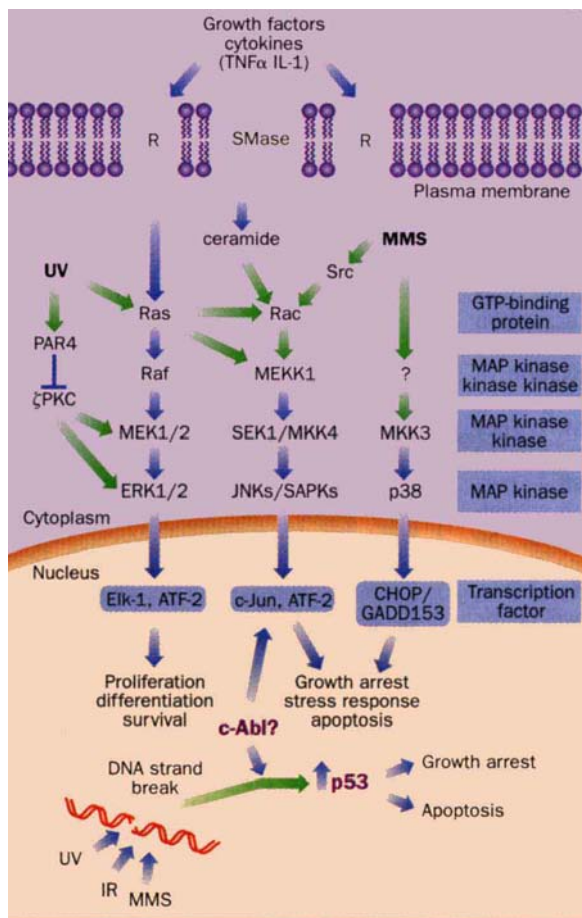
Christine E. Canman and Michael B. Kastan

CELLULAR stresses elicit biochemical responses that either enhance cell survival or lead to cell death. For example, SOS pathways in bacteria seem to increase DNA repair in response to DNA damage; however, the induction of p53 by DNA damage in certain mammalian cells can facilitate apoptotic cell death. The existence of an SOS-type system that leads to

that the specific combination of pathways that is induced by a particular genotoxin in a particular cell type dictates the fate of the cell after genotoxic stress. And it is intriguing that growth factors, which provide survival signals for cells, activate many of the same gene products as these genotoxic stresses.

Several different mammalian, genotoxin-inducible pathways have attracted attention in recent years, involving proto-oncogene products, such as the c-Jun kinases and c-Abl, and tumour-suppressor gene products, such as p53. The c-Jun amino-terminal kinases (JNKs), also referred to as stress-activated protein kinases (SAPKs), are activated in response to inflammatory cytokines and a variety of genotoxic stresses, including treatment with alkylating agents, ultraviolet light and ionizing radiation. The p53 tumour-suppressor gene product is induced by a number of DNA-damaging agents, and p53 is involved in both growth arrest and apoptotic cell death². The c-Abl tyrosine kinase has been linked to DNA-damage-inducible pathways, and not only appears to interact with p53 and the retinoblastoma protein, but also participates in the activation of JNKs in cells that have been treated with various DNA-damaging agents³⁻⁵.

Although we typically think of DNA as the target of genotoxins, these agents also frequently activate pathways that originate in the cell membrane or cyto-



Common features of signal-transduction pathways activated by growth factors, inflammatory cytokines and genotoxic agents. Green arrows represent provisionally understood steps. R, receptor; SMase, sphingomyelinase; IR, ionizing radiation; UV, ultraviolet light; MMS, methyl methane-sulphonate.

enhanced DNA-damage-inducible repair in mammalian cells has been suggested, but such a pathway has not been clearly demonstrated.

On page 273 of this issue, Liu and co-workers¹ describe studies of the responses of cells to various genotoxic agents, and they find that these agents can activate several different pathways—the c-Abl, JNK or p53 pathways. Different agents activate the c-Abl and JNK pathways to differing extents, but p53 responds to every agent tested, independently of c-Abl or JNK, and stands alone as the “universal sensor of genotoxic stress”¹. It is possible

plasm, triggering similar phosphorylation cascades to those induced by the activation of cell-surface receptors. Activation of three distinct, but related, pathways culminates in the selective activation of the three different sets of mitogen-activated protein (MAP) kinases (see figure): p42 and p44 (also known as extracellular-signal-related kinase (ERK)-1 and ERK2), JNK1 and JNK2, and p38 (Mpk2, the mammalian homologue of HOG1 from yeast)⁶. These kinases translocate into the nucleus where they selectively activate several different transcription factors^{6,7}. ERKs are primarily activated in

1. Lutz, D. et al. *Astron. Astrophys.* **315**, L137–L140 (1996). Refs 1–4 also available at: <http://isowww.estec.esa.nl:80/ISO/AandA>
2. Vigroux, L. et al. *Astron. Astrophys.* **315**, L93–L96 (1996).
3. Helmich, F. P. et al. *Astron. Astrophys.* **315**, L173–L176 (1996).
4. Abergel, A. et al. *Astron. Astrophys.* **315**, L329–L332 (1996).
5. Sanders, D. & Mirabel, I. *Annu. Rev. Astron. Astrophys.* (in the press).

response to growth-factor stimulation, and they have been extensively characterized within the Ras/Raf/MEK/ERK cascade. JNKs, on the other hand, can participate in growth-factor signalling, as well as in many overlapping stress responses that activate p38.

How do genotoxic agents selectively trigger MAP-kinase pathways in the cytoplasm? In the case of growth factors, extracellular signals are transduced at the plasma membrane by the recruitment of the GTP-binding protein Ras to tyrosine-phosphorylated receptors. Ras then relays this signal to a MAP kinase kinase kinase (Raf). Raf activates the next participant, a MAP kinase kinase (MEK), which in turn activates a MAP kinase (ERK). This pattern of three activating kinases is preserved in all three of the MAP-kinase pathways.

Karin and co-workers⁸ previously reported that the stimulation of the transcription factor AP-1 by short-wavelength ultraviolet radiation depends on the activities of Ras, Raf and the tyrosine kinase Src. Liu *et al.*¹ now report that Src does not seem to be required for JNK activation after exposure to ultraviolet light, but that Src does contribute to the activation of JNK in response to the alkylating agent methyl methanesulphonate (MMS). They also provide evidence that the Ras-related protein, Rac — but not Ras itself — participates in the pathway, selectively activating JNK in response to MMS. So ultraviolet light and MMS activate distinct targets that are upstream of Ras and Rac, and the identity of these targets will be the focus of future research.

What are the physiological consequences of JNK activation in response to genotoxic agents? JNKs have been associated with cell death^{9,10}, and it is possible that cell survival and death are dictated by a balance between ERK and JNK signalling¹¹. Two atypical isoforms of protein kinase C, λ PKC and ζ PKC, have joined the collection of proteins involved in maintaining this balance between survival and death. These PKCs are 'atypical' because they are regulated by ceramide and phosphatidylinositol 3,4,5-triphosphate (generated by phosphatidylinositol-

3 kinase), rather than by diacylglycerol or phorbol esters. Atypical PKCs are involved in both mitogenic and survival signalling because they are targets of phosphatidylinositol-3 kinase and they activate the Ras/Raf/ERK signalling cascade¹¹⁻¹⁴. The ζ PKC isoform is inhibited by a recently identified gene product, PAR4, which is induced by ultraviolet light and promotes apoptosis¹⁵.

A common activator of the three MAP-kinase pathways may be ceramide. Ceramide can stimulate the ERK pathway by enhancing the activities of ζ PKC and ceramide-activated protein kinase — the latter phosphorylates and activates Raf (ref. 16). Ceramide also activates the JNK pathway, and it has been implicated in inducing apoptosis in response to ionizing radiation and other stresses^{10,17}. Here again, a balance between apparently opposing signalling pathways may dictate the cellular outcome.

Another grey area is the role of c-Abl in modulating these responses. Liu *et al.*¹ found that JNK activation was not as

dependent on the presence of c-Abl as a previous study had shown⁵, even though the same cell lines were used. It has also been suggested that c-Abl cooperates with p53 to downregulate cyclin/cdk2 activity in the p53 signal-transduction pathway³. But Liu *et al.* argue that c-Abl is unlikely to contribute to p53-dependent G1 arrest because c-Abl is only activated during S phase. Further experiments are clearly required.

In the absence of a better explanation, we often state that cellular outcome following exposure to genotoxic agents is dependent on cellular context. An obvious goal is to gain a better understanding of the molecular effectors that interact to determine whether a cell survives or dies following the activation of these distinct signalling pathways. □

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Spot the referee



A VENERABLE practice in British newspapers, now largely out of fashion, is the spot-the-ball competition. An action photograph from (usually) a soccer match is doctored so that the ball does not appear in the published picture. Readers are invited to guess the position of the ball's centre by marking a cross on the picture, and the winner is rewarded with a modest prize.

Inspired by a brief paper in the *British Medical Journal* (313, 1185; 1996), News and Views offers a spot-the-referee competition. The paper, by S. Wessely and colleagues, addresses the question that exercises anyone who has submitted a paper to a peer-reviewed journal and has had it returned with reviewers' comments: "Who refereed my paper?"

Over a five-month period, Wessely *et al.* asked authors who had submitted papers to *Psychological Medicine* whether, on seeing the referees' reports, they could identify the referee. Out of the 252 responses they received, in

only 15 instances was the referee correctly identified; 36 were incorrectly identified, and in the remaining cases the author had no clue as to who the referee was. Wessely *et al.* conclude that, even when a specialist journal is concerned, the sometimes elaborate attempts to guess who has refereed a paper are "largely unrewarding".

This journal does not intend to carry out a similar exercise, but readers are offered the diversion of spotting the position of the referee whose image has been removed from this photograph. His nose is the target part of his anatomy, and should be indicated in x,y coordinates, in millimetres, the bottom left-hand corner of the picture being the origin (e-mail: nature@nature.com; closing date 5 December). The prize at stake is a year's subscription to *Nature*; if there is more than one correct entry (accurate to within 0.5 mm in both coordinates) the winner will be chosen by a random draw. □

1. Lui, Z.-G. *et al.* *Nature* **384**, 273-276 (1996).
2. Ko, L. J. & Prives, C. *Genes Dev.* **10**, 1054-1072 (1996).
3. Yuan, Z. M. *et al.* *Nature* **382**, 272-274 (1996).
4. Welch, P. J. & Wang, J. Y. J. *Cell* **75**, 779-790 (1993).
5. Kharbanda, S. *et al.* *Nature* **376**, 785-788 (1995).
6. Su, B. & Karin, M. *Curr. Opin. Immunol.* **8**, 402-411 (1996).
7. Wang, X. & Ron, D. *Science* **272**, 1347-1349 (1996).
8. Devary, Y., Gottlieb, R. A., Smeal, T. & Karin, M. *Cell* **71**, 1081-1091 (1992).
9. Ham, J. *et al.* *Neuron* **14**, 927-939 (1995).
10. Verheij, M. *et al.* *Nature* **380**, 75-79 (1996).
11. Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J. & Greenberg, M. E. *Science* **270**, 1326-1331 (1995).
12. Akimoto, K. *et al.* *EMBO J.* **15**, 788-798 (1996).
13. Berra, E. *et al.* *EMBO J.* **14**, 6157-6163 (1995).
14. Yao, R. & Cooper, G. M. *Science* **267**, 2003-2006 (1995).
15. Diaz-Meco, M. T. *et al.* *Cell* **86**, 777-786 (1996).
16. Yao, B. *et al.* *Nature* **378**, 307-310 (1995).
17. Santana, P. *et al.* *Cell* **86**, 189-199 (1996).

Reading garnet's signature

Marc Hirschmann

IN the quest to understand the genesis of oceanic crust, a central observation is that crust formation is not equally vigorous everywhere. In Iceland, where the mid-ocean ridge emerges above sea level, new crust is more than 15 km thick. At the other extreme, some ridges lie beneath 5,000 m of water or more, and have crust less than 4 km thick. Is this variation in crust thickness caused primarily by variation in the temperature of upwelling mantle, or by varying extents of cooling from the surface? And what effects might differences in mantle composition have?

To answer these questions, we must know the depth at which melting begins in mid-ocean-ridge basalt (MORB) source regions. On page 231 of this issue¹, Bourdon *et al.* report uranium-series radioisotope data from MORB that indicate that melting begins at greater depths beneath 'shallow' ridges (that is, those with thick crust), and they therefore propose that temperature differences between MORB source regions are a primary cause of variations in oceanic crust production. But the new data seem to run counter to the results of two other studies^{2,3}, and the discrepancy creates an important new puzzle.

Bourdon *et al.* report the first worldwide compilation of $(^{230}\text{Th})/(^{238}\text{U})$ ratios for MORB (the parentheses signify abundance multiplied by the nuclide's decay constant). Garnet is the key here — basalts with $(^{230}\text{Th})/(^{238}\text{U})$ greater than unity require mantle sources that contain garnet^{4,5}, which is stable only in the deeper portions of MORB source regions (Fig. 1). Bourdon *et al.* find that the magnitude of regionally averaged $(^{230}\text{Th})/(^{238}\text{U})$ is negatively correlated with water depth. This is best explained if the initial depth of melting is greatest for shallow ridges, allowing garnet–melt interaction over a larger part of the melting and melt-transport regime (although this interpretation requires a number of assumptions; see the caption to Fig. 2). As shown in Fig. 2, a logical interpretation is that the mantle beneath shallow ridges is hotter than that beneath deep ridges, although the data do not rule out some influence from variations in mantle composition.

This work builds on earlier studies that showed that global variations in major-element compositions of MORB are consistent with increased depth of initial melting and increased temperature beneath shallower ridges^{6,7}. Seismic velocity data also suggest that mantle temperatures are inversely proportional to ridge depth^{8–10}, and so there are several lines of evidence linking crustal thickness to temperature variation.

However, the new data¹ are seemingly in conflict with the results of two other studies of the role of garnet in MORB petrogenesis. Ratios of samarium (Sm) to ytterbium (Yb), and the isotope systematics of neodymium (Nd) and hafnium (Hf), are also believed to be indicators of garnet in mantle source regions. A compilation of Sm/Yb data by Shen and Forsyth², and

of melting, whereas Sm/Yb and Nd–Hf garnet signatures seemingly require volumetrically significant amounts of garnet-equilibrated melt. Second, U-series disequilibria are more sensitive than Sm/Yb and Nd–Hf isotopes to rates of melting and melt transport. If peridotite upwelling is more rapid beneath regions of thick crust than beneath thin crust, as has been suggested^{2,3}, then $(^{230}\text{Th})/(^{238}\text{U})$ should be affected more than Sm/Yb or Nd–Hf systematics. However, most models of generation of $(^{230}\text{Th})/(^{238}\text{U})$ predict that ^{230}Th excesses should be enhanced

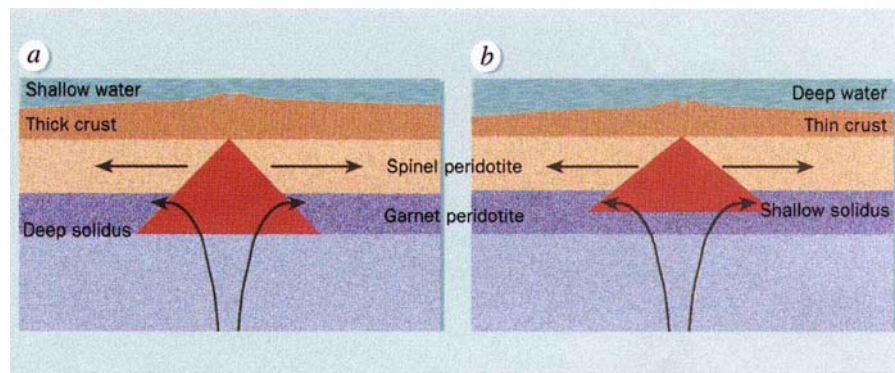


FIG. 1 Differences between melting regimes beneath mid-ocean ridges in shallow waters (which have thick crust) and those in deep waters (thin crust), as inferred from the $(^{230}\text{Th})/(^{238}\text{U})$ data of Bourdon *et al.*¹. Red regions are where upwelling mantle undergoes melting; mauve shows regions where garnet is present. As plates pull apart at mid-ocean ridges, mantle upwells and begins to melt at greater depths beneath shallow ridges (a) than beneath deep ridges (b). Melts generated deep in the former regime traverse a greater distance in the presence of garnet peridotite than those generated deep in the latter, which leads to larger $(^{230}\text{Th})/(^{238}\text{U})$ in a (ref. 12).

Nd and Hf isotope data of Salters³, both show weaker garnet signatures for regions of thick crust and stronger ones for regions of thin crust. These results imply that variable depth of melting is not one of the main determinants of crust thickness.

Instead, Shen and Forsyth² inferred that, except beneath areas of very thick crust, variations in the depth at which melting ceases are more influential. Thus, beneath regions of thin crust, melting should cease at greater depths and proportionally larger amounts of melt should form by partial melting of garnet peridotite. But shallow termination of melting cannot explain the large ^{230}Th excesses that Bourdon *et al.* find is characteristic of regions of thick crust, which presents a serious challenge to Shen and Forsyth's interpretation. On the other hand, the Sm/Yb and Nd–Hf observations cannot be explained by lateral variations in temperature, and the variable-temperature model will at the very least require elaboration.

The conflicting evidence cannot be fully resolved at this time, but there are a number of considerations to bear in mind. First, the different garnet signatures come from elements with distinct geochemical behaviour; U and Th are much less compatible than the rare-earth elements or Hf. So $(^{230}\text{Th})/(^{238}\text{U})$ in MORB can be dominated by processes at the very onset

by slow peridotite upwelling rates^{11,12}, which is not consistent with greater $(^{230}\text{Th})/(^{238}\text{U})$ observed at regions of thick crust. Third, inferences about the role of garnet from Sm/Yb and Nd–Hf isotope systematics depend on assumptions about the composition of the source region, but those from $(^{230}\text{Th})/(^{238}\text{U})$ do not. Some portion of the garnet signatures inferred from Sm/Yb and Nd–Hf systematics could be caused by variable mantle composition.

One explanation for the apparently conflicting garnet signatures is that $(^{230}\text{Th})/(^{238}\text{U})$ could be dominated by small degrees of dehydration melting of peridotite at great depth¹³, and the Sm/Yb and Nd–Hf isotope systematics could be dominated by processes at shallower depths. One such might be partial melting of garnet pyroxenite veins, as garnet is stable to relatively low pressures in pyroxenite¹⁴. If dehydration melting occurs deeper in regions of thick crust, either because of higher temperatures or higher volatile contents, the trend observed by Bourdon *et al.* should result. Because partial melts from garnet pyroxenite should be more diluted in regions of thick crust, their effect on Sm/Yb and Nd–Hf might be greatest in regions of thin crust. Also, garnet pyroxenite should have different trace-element compositions from those of typical mantle peridotite¹⁴, so some of the Sm/Yb and Nd–Hf systematics inferred to

Resolving a paradox of pain

Kenneth L. Casey

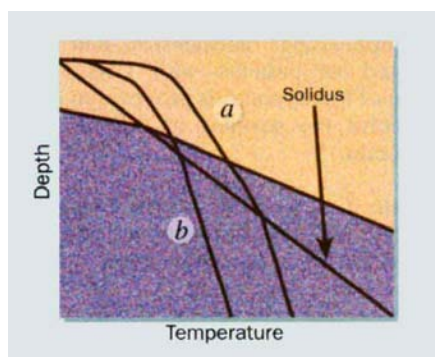


FIG. 2 The difference between the two situations depicted in Fig. 1 can be explained if mantle upwelling beneath *a* is hotter than that beneath *b*, as hotter mantle will intersect the peridotite solidus at greater depth. This model depends on some assumptions, however. First, the melts, once formed, must migrate upwards more rapidly than the upwelling solid matrix and, at least initially, must continue to equilibrate with the matrix as it migrates. Second, at least some portion of the migrating melts must continue to equilibrate with the matrix until they reach the height of garnet exhaustion. Alternatively, the distance over which melts continue to equilibrate with garnet-bearing matrix should be proportional to the depth of initial melting. Although the melting regimes depicted here can explain variations in $(^{230}\text{Th})/(^{238}\text{U})$, they do not explain other observations^{2,3} (as discussed in the main text).

be garnet signatures could instead derive from mantle heterogeneity.

Clearly, however, we do not fully understand garnet signatures in MORB. A complete explanation will probably have to take into account variations in temperature and mantle composition, and possibly the style and rate of upwelling. For now, the correlation between $(^{230}\text{Th})/(^{238}\text{U})$ and ridge depth identified by Bourdon *et al.* provides a critical constraint on models of the processes that create oceanic crust. □

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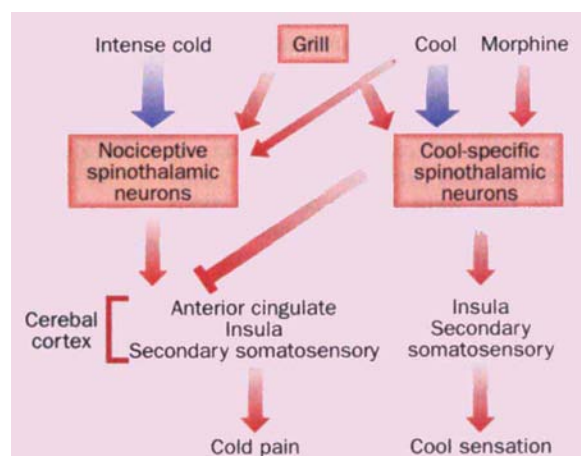
PARADOXES and illusions sometimes lead to useful insights into the basic mechanisms that underlie the function — and malfunction — of complex systems. This is elegantly shown by Craig *et al.*¹ on page 258 of this issue, using an unusual illusion of pain to model the neurological events that take place in some diseases of the central nervous system.

The paradox is presented by a problem that regularly confronts clinical neurologists or neurosurgeons when they attempt to treat patients who have pain that is caused by a disease or trauma that directly affects the central nervous system. Known as central pain syndromes (CPS)², these affect a significant minority of patients suffering from stroke, trauma, multiple sclerosis, and other disorders of the brain or spinal cord. Typically, the patient complains of nearly constant deep aching or burning pain that is distributed within the neurologically impaired body area. The paradox is that, compared with people who do not have CPS, nearly all of these patients have a reduced ability to detect noxious stimulation when it is applied to the neurologically impaired body area. They are also unable to detect innocuous temperature sensations within the same area. In some patients with CPS, the thermal sensory loss is the predominant

or only symptom. Others may also have allodynia ('different pain'), which is an unusual sensitivity to normally innocuous stimulation, so that touches or mild temperatures are perceived as being painful when applied within the neurologically impaired area³.

The brain mechanisms that mediate the sensations of temperature discrimination and pain depend on direct impulses between the spinal cord and several anatomically and functionally distinct neurons in the thalamus, which communicates with several distinct areas of the overlying cerebral cortex. Many of the neurons that form this spinothalamic tract are nociceptive — they respond either exclusively or differentially to noxious mechanical, chemical or thermal stimulation⁴. Craig and Hunsley showed through neurophysiological studies⁵ that some spinothalamic neurons also respond to innocuous cool stimuli. After systemic treatment with

morphine, the discharges of these 'cool' cells increased, in contrast to most nociceptive spinothalamic neurons, in which the discharges decreased in response to treatment with morphine. This was one of the first indications that the pathways activated by cool stimuli might be involved in the normal modulation of the excitability of spinothalamic tract cells. Now Craig *et al.*¹ have connected these observations of the nociceptive and cool spinothalamic neurons, and have used them to formulate a new and specific hypothesis about the pathophysiology of CPS.



How the thermal grill might produce an illusion of pain in the experiments done by Craig *et al.*¹. Although cool stimuli can excite nociceptive spinothalamic cells, they do not produce pain because of the suppressive effect (site of action unknown) of the strongly activated, cool-specific spinothalamic neurons. The grill stimulus has a similar effect, but it is less excitatory to the cool-specific cells, so it reduces their suppressive effect on cold pain. Morphine may also activate this pain-suppressive system.

To do this, Craig *et al.* used a 'thermal grill' — an illusion in which alternating warm and cool bars produce a sensation similar to the burning pain of intense cold. The grill provides a cutaneous stimulus that causes normal human subjects to feel pain without stimulating the nociceptive cutaneous receptors. In other words, this mimics the thermal sensitivity experienced by some CPS patients, and suggests that there is normally an interaction between the neural mechanisms that mediate pain and those that detect the sensations of innocuous warm and cool. So finding a neurophysiological basis for the grill illusion could provide important clues to the pathophysiology of the abnormal sensations in CPS.

Previous neurophysiological studies in animals showed that, while innocuous cool stimuli had a strong excitatory effect on cool-specific spinothalamic cells and a weak effect on their nociceptive counter-

1. Bourdon, B., Zindler, A., Elliott, T. & Langmuir, C. H. *Nature* **384**, 231–235 (1996).
2. Shen, Y. & Forsyth, D. W. *J. Geophys. Res.* **100**, 2211–2237 (1995).
3. Salters, V. J. M. *Earth Planet. Sci. Lett.* **141**, 109–123 (1996).
4. Beattie, P. *Nature* **363**, 63–65 (1993).
5. LaTourrette, T. Z., Kennedy, A. K. & Wasserburg, G. J. *Science* **261**, 739–742 (1993).
6. Klein, E. M. & Langmuir, C. H. *J. Geophys. Res.* **92**, 8089–8115 (1987).
7. Dick, H. J. B., Fischer, R. & Bryan, W. B. *Earth Planet. Sci. Lett.* **69**, 88–106 (1984).
8. Zhang, Y. S., Tanimoto, T. & Stolper, E. M. *Phys. Earth Planet. Inter.* **84**, 79–93 (1994).
9. Langmuir, C. H. *Nature* **364**, 191–192 (1993).
10. Humler, E., Thiriot, J. L. & Montagner, J. P. *Nature* **364**, 225–228 (1993).
11. McKenzie, D. *Earth Planet. Sci. Lett.* **72**, 149–157 (1985).
12. Spiegelman, M. & Elliott, T. *Earth Planet. Sci. Lett.* **118**, 1–20 (1993).
13. Hirth, G. H. & Kohlstedt, D. L. *Earth Planet. Sci. Lett.* **144**, 93–108 (1996).
14. Hirschmann, M. M. & Stolper, E. M. *Contrib. Mineral. Petrol.* **124**, 185–208 (1996).

parts, the 'thermal grill' stimulus had an equally excitatory effect on the nociceptive spinothalamic neurons and the 'cool' spinothalamic cells⁶ (see figure). These findings, and the observations of others^{7,8}, suggested that the grill illusion of pain could be produced by a reduction in the activity of spinothalamic 'cool' cells that normally attenuate the pain produced by the concurrently active nociceptive spinothalamic neurons.

If the grill produces pain by stimulating the nociceptive spinothalamic cells that respond to noxious cold, then, in normal humans, it should activate the cerebral structures that usually respond to noxious stimuli. In other words, the pattern of cerebral activation will resemble that of the response to noxious-cold stimulation; and this is the result obtained by Craig *et al.* Using positron-emission tomography, they identified pain-related increases in regional cerebral blood flow, caused by increased synaptic activity. The grill and noxious-cold stimulation produced identical patterns of cerebral activation; in agreement with previous studies⁹⁻¹², only stimuli that were perceived as noxious activated the anterior cingulate cortex, together with the insular and secondary somatosensory cortices. This result highlights the importance of the anterior cingulate in pain perception, and it is consistent with the hypothesis that the grill attenuates the excitation of spinothalamic 'cool' cells, allowing the activity of nociceptive spinothalamic neurons that respond to cold stimuli to be unmasked.

Although there is psychophysical evidence that innocuous warm stimuli can attenuate heat pain sensation¹³, a population of 'warm' spinothalamic neurons with properties corresponding to those of the spinothalamic 'cool' cells has not been identified. Furthermore, both warm and painfully hot stimuli produce a pattern of cerebral activity, which, unlike either the grill or noxious cold, includes the primary somatosensory cortex.

The spinothalamic 'cool' and nocicep-

tive pathways must converge for one to inhibit the other, and the study by Craig *et al.*¹ does not identify the site of this interaction. But by combining an illusion of pain with information obtained from psychophysical, neurophysiological and functional cerebral-imaging studies, they have proposed a potentially testable hypothesis — that neuronal impulses generated by cool stimuli and transmitted

through the spinothalamic tract, activate pain-suppression mechanisms that are inhibited in patients with CPS. The testing of this hypothesis promises to help to resolve the paradox of central pain syndromes. □

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RNA VIRUSES

Life on the edge of catastrophe

Stuart Nichol

A FANTASTIC picture of RNA virus life on the edge of error catastrophe emerged at the recent meeting on RNA viral quasispecies in Madrid, Spain* — a picture that Salvador Dali himself would have enjoyed, with everything from molecular-clock distortions and Red Queens to mutational meltdowns and bottlenecks.

RNA viruses encode the RNA poly-

merase, (P. Minor, NIBSC, Potters Bar, UK). The inherent error-rates of these types of virus are so high that the treatment of VSV with mutagens only modestly increases the single-site mutation rates. However, this small (1.3–2-fold) elevation in the mutation rate leads to a decrease in the adaptability of the virus (J. Holland, Univ. California, San Diego).

In other words, at such high polymerase error-rates, further increases throw these viruses into a state of 'mutational meltdown', where the information within the genome becomes essentially scrambled.

Many factors can increase or decrease the fidelity of RNA polymerases, including the substitution of critical amino-acid residues in the polymerase active sites, and alteration of the relative ratios of individual nucleoside triphosphates (S. Wain-Hobson, Pasteur Inst.; L. Loeb, Univ. Washington; L. Menendez-Arias, Univ. Autonoma, Madrid).

In extreme cases, the polymerase is so error-prone that hypermutation can be seen, where many substitutions — particularly A to G — occur in a single replication cycle (J. Molero, Centro Nacional de Biología Fundamental, Madrid). This may be bad news with regard to the long-term effectiveness of some antiviral compounds and vaccines, but it may be good news for evolutionary biotechnology. Here, hypermutation can be exploited to explore large areas of sequence space in search of biologically useful molecules. The fate of a specific mutant will depend on the spectrum of mutants that surrounds it, so the virus that grows the most rapidly (or the fastest-replicating RNA) may not always dominate the population (C. Biebricher, Max-Planck-Inst. Biophys. Chem., Göttingen-Nicolausberg; J. Holland; M. Eigen, Max-Planck-Inst. Biophys. Chem.).

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Catastrophic consequences — human influenza type A viruses are spread in droplets from coughs and sneezes. Tiny spikes on the viral protein coats may change in structure to create new influenza strains that can spread as an epidemic.

merases that are used to replicate their genomes. Because of the high polymerase error-rates (approximately 10^{-3} to 10^{-4} substitutions per base) and a lack of proof-reading mechanisms, these viruses exist in the form of quasispecies (a swarm of genetic microvariants). Mutations are introduced into the viral genomes as they replicate, and several examples of viruses with high mutation rates were presented at the meeting, including human immunodeficiency virus (HIV), human influenza virus A (see figure), vesicular stomatitis virus (VSV), foot and mouth disease virus (FMDV) and polioviruses. Some viruses also have high recombination rates, for example HIV (D. Richman, Univ. Califor-

*International workshop on RNA Viral Quasispecies, Instituto Juan March de Estudios e Investigaciones, Madrid, Spain, 7–9 October 1996.

1. Craig, A. D., Reiman, E. M., Evans, A. & Bushnell, M. C. *Nature* **384**, 258–260 (1996).
2. Casey, K. L. (ed.) in *Pain and Central Nervous System Disease* (Raven, New York, 1991).
3. Boivie, J., Leijon, G. & Johansson, I. *Pain* **37**, 173–185 (1989).
4. Willis, W. D. *The Pain System: The Neural Basis of Nociceptive Transmission in the Mammalian Nervous System* (Karger, Basel, 1985).
5. Craig, A. D. & Hunsley, S. J. *Brain Res.* **558**, 93–97 (1991).
6. Craig, A. D. & Bushnell, M. C. *Science* **265**, 252–255 (1994).
7. Yaritsky, D. & Ochoa, J. L. *Brain* **113**, 893–902 (1990).
8. Bini, G., Cruccu, G., Hagbarth, K.-E., Schady, W. & Torebjörk, E. *Pain* **18**, 239–248 (1984).
9. Jones, A. K. P., Brown, W. D., Friston, K. J., Qi, L. Y. & Frackowiak, R. S. J. *Proc. R. Soc. Lond. B* **244**, 39–44 (1991).
10. Casey, K. L. *et al.* *J. Neurophysiol.* **71**, 802–807 (1994).
11. Coghill, R. C. *et al.* *J. Neurosci.* **14**, 4095–4108 (1994).
12. Casey, K. L., Minoshima, S., Morrow, T. J. & Koeppe, R. A. *J. Neurophysiol.* **76**, 571–581 (1996).
13. Casey, K. L., Zumberg, M., Heslep, H. & Morrow, T. J. *Somatosens. Motor Res.* **10**, 327–337 (1993).

DAEDALUS

Predicting the genetic stability and fitness of a virus during serial tissue-culture passage is a complex problem, and depends on the starting fitness of the virus, the size of the virus population that is passed, and the host-cell environment. Consistent with Muller's ratchet, bottlenecks — serial passage (or plaque-to-plaque transfer) of low numbers of a virus with high starting fitness — results in reduced fitness, to the point that after several such passes, recovery of infectious virus becomes difficult. This has been shown for VSV (J. Holland) and for FMDV (C. Escarmis, Univ. Autònoma, Madrid). Conversely, serial transfer of a large virus population of low fitness can lead to a rapid increase in fitness. Just like a couple of well-matched poker players, if two viruses of equal starting fitness are placed in competition, one eventually wins and out-competes the other. But during the competition both the winner and the loser show fitness gains. This is in agreement with the predictions of two major theories of classical population biology, the competitive exclusion principle and the Red Queen's hypothesis — named after the Red Queen in *Alice Through the Looking-Glass* who pronounced "it takes all the running you can do to keep in the same place".

A common question in evolutionary biology is whether group or individual selection operates in a particular system. Experimental and theoretical evidence indicates that group selection operates in RNA-virus systems, the target of selection being the entire quasispecies rather than individual virus genomes (J. Holland; M. Eigen). Frequency-dependent selection has also been shown in several systems. In other words, the outcome of competition between two viruses will depend on their relative frequencies in the starting population.

The big challenge in the field is in trying to use the base of knowledge from model and theoretical systems to understand infectious diseases in animals and plants. RNA viruses can quickly exploit new ecological niches and jump between host species, and these may be important reasons why many of the emerging and re-emerging diseases over the past few decades have been due to RNA viruses. But not all RNA viruses evolve rapidly in nature: VSV, Ebola virus and hantaviruses all seem to accumulate mutations relatively slowly, indicating the importance of constraining selective forces (B. Mahy and S. Nichol, CDC, Atlanta).

One area that is receiving much attention is the generation of genetic diversity in HIV, and its importance in the progression of AIDS and the development of resistance to antiviral drugs. Despite the importance of T cells in restricting HIV replication, there is no clear evidence that T-cell recognition of infected cells is a selective force that drives the evolution of

HIV. Either such an effect is absent, or it is masked by the fact that T-cell recognition is oligoclonal — various epitopes of several HIV proteins are recognized at any one time, even in individual patients (A. Meyerhans, Univ. Freiburg). In spite of earlier suggestions, there has been no evidence from studies of a chimaeric simian/HIV-macaque model for AIDS that HIV-1 Env protein acts as a super-antigen for T-cell activation (N. Letvin, Harvard Med. School).

The genetic complexity of the HIV microvariant pool in the blood is highly dynamic because of the differing contributions made by independently evolving virus subpopulations that replicate at many sites in an infected patient (D. Richman). Even non-HIV-related, localized antigenic stimulation of latently infected cells can result in the rapid increase in the replication of an HIV subpopulation and in its relative contribution to the virus load (S. Wain-Hobson; A. Meyerhans).

Surprisingly, temperature-sensitive *Escherichia coli* mutants that contain defective DNA polymerase I can be complemented by HIV reverse transcriptase (L. Loeb). This provides a powerful system for the analysis of HIV reverse transcriptase mutants and inhibitors. Drug-resistant HIV mutants quickly arise when patients are treated with individual antiviral agents such as AZT, 3TC or protease inhibitors. But every chameleon has its limits, and a cocktail of all three antivirals seems to be promising in arresting HIV replication and preventing the appearance of resistant mutants in HIV-infected patients (D. Richman). Other limitations to the diversity of viable RNA viruses may exist. For instance, there may be some limit to the spectrum of viable antigenic variants possible for viruses such as influenza A virus, FMDV or poliovirus, all of which have cell-receptor-recognition regions that are in close proximity to neutralization epitopes (E. Domingo, Univ. Autònoma, Madrid; E. Wimmer, State Univ. New York at Stony Brook; J. Skehel, NIMR). This is particularly true for FMDV and polioviruses, which have the additional constraint of highly ordered capsid structures. Similarly, the evolution of the V3 domain of HIV-1 subtype-B viruses seems to be confined to an area in sequence space within a fixed distance from the subtype-B consensus sequence (J. Goudsmit, Univ. Amsterdam).

By the end of the meeting, there was a sense that RNA viruses enjoy life in the evolutionary fast lane; however, we slow-moving, DNA-based life forms may have some opportunities to outwit them yet. □

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Size and shape

THE clothing industry has one major problem: people vary in size. It has to produce each style of garment in many different sizes, and must guess how many of each to make. Every wrong guess loses money. Daedalus now has a way out.

He recalls the phenomenon of plastic memory. When a suitable polymer is heated, shaped and allowed to cool, it retains that shape. If warmed up again, it contracts to its original form, from which it can be reshaped again. DREADCO chemists are now conferring this property on synthetic fibres. A garment woven from such a fibre could be heated to its plastic-memory temperature, and stretched to take any desired shape or size. When cooled down, it would hold that form. It would not cling to its wearer like a conventional elastic garment, but would have the drape and feel of normal loose clothing. Yet a shop could stock just one size of each style. With the aid of an adjustable heated former, this could be shaped to fit any customer; and reshaped again later if the fit seemed wrong.

Pure synthetics, however, are unpopular. People prefer mixed fabrics containing wool or cotton as well. The DREADCO team are devising threads in which cotton fibres (for example) are helically wound or spun round a synthetic core, so that they stretch or shrink with it. Only a very uniform fibre will give a fabric that stretches and shrinks evenly, with no unsightly loose or buckled regions, but the project seems feasible.

The rag trade will be revolutionized. Manufacturers will make one small size only, and shops will stretch it to fit each customer. Even better, old but loved garments that no longer fit could be endlessly readjusted; in particular, children will never (or hardly ever) grow out of their clothes. And fashion will lose its terrors. If Paris decrees that skirts will be longer or shorter this season, or lapels more prominent, followers of fashion will no longer be forced to buy a whole new wardrobe. They will simply adjust their existing clothes to the new standard of chic.

At first Daedalus feared that bespoke tailors would be driven out of business. But he now reckons that they will still retain their traditional rich, vain clientele. For the visible woven or printed pattern on a plastic-memory suit or dress will expand with the garment where it is locally stretched. It will subtly betray the fact that the wearer has a paunch or posterior far larger than the minimum envisaged by the designer. A traditionally tailored suit, expensively handcrafted from cloth of dimensionally stable pattern, will usefully conceal this unwelcome fact.

David Jones

Geoffrey Wilkinson (1921–96)

"He has made more isotopes of the chemical elements than any other human being." So spoke Glenn Seaborg in 1976 as he conferred the Centennial Foreign Fellowship of the American Chemical Society upon Geoffrey Wilkinson, who died suddenly in his home in London on 26 September. But this was by no means Wilkinson's only claim to chemical fame.

He prepared a compound of rhodium, later known as Wilkinson's catalyst, which smoothly effects the hydrogenation of numerous compounds into valuable pharmaceutical products. He devised another compound of rhodium that is used in the industrial process of hydroformylation, whereby olefins are converted with 'syngas' (a mixture of CO and H₂) to aldehydes and alcohols. And in 1952, in the Chemistry Library of Harvard University, at about 4 pm on 30 January — his *tempus mirabile* — he achieved what many believe to be his greatest act of imaginative insight: he divined a rational structure for a novel organic compound of iron, Fe(C₅H₅)₂, that had been discovered by P. L. Pauson at Duquesne University (T. J. Kealy & P. L. Paulson *Nature* 168, 1039–1040; 1951), where an atom of iron is sandwiched between two planar cyclopentadienyl moieties. (Astonishingly, another Harvard chemist, R. B. Woodward, had arrived independently at the same structure, at about the same time.) It was for his "pioneering work on the chemistry of the organometallic so-called sandwich compounds" that Wilkinson won the Nobel prize for chemistry, which he shared with E. O. Fischer of Munich in 1973.

He was born in Yorkshire in 1921 and educated at Todmorden Secondary School (Sir John Cockcroft, Nobel laureate in physics, 1951, was also educated there). He won a royal scholarship to Imperial College, London, and graduated top of his year in 1941. By then, the Second World War was underway, and along with some of the brightest scientific minds of the day, he was recruited for the joint UK/US/Canadian atomic energy programme. They sailed from Greenock for Canada on 11 January 1943 aboard the RMS *Andes*, surviving the hazards of the North Atlantic.

From 1943 to 1946 he worked at Montreal and at Chalk River, developing skills in nuclear chemistry. At the end of hostilities, he joined Glenn Seaborg's research group at Berkeley, where, from 1946 to 1951, during his prodigious work on new isotopes, he accomplished the alchemist's dream: he transmuted another element into gold.

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After his move east to MIT and then to Harvard in 1950, Wilkinson left nuclear chemistry and entered mainstream inorganic chemistry. Between 1950 and 1955, he developed a flair and a *modus operandi* for his research that he sustained for the next forty years. At Harvard, he realized that the 'sandwich' structural concept should be extendible to other metals and ligands, and there emerged over the next three years a series of now classic papers on these new aspects of organometallic chemistry.

In January 1956 he returned to Imperial College, London, to the then only established chair of inorganic chemistry in Britain. He was 34, and one of the youngest professors ever appointed by the college. For the next forty years he was to maintain a remarkable output of outstanding chemistry. He gathered around him and inspired a group of co-workers who raced towards establishing the fundamentals of transition-metal organometallics. Many of his scientific progeny now occupy major posts across the continents.

His shrewdness and remarkable intuitive skills drove his chemistry into ever-new pastures. He had the knack of picking good graduate students and postdoctoral workers whom he led in the laboratory by example, and from whom he demanded the highest scientific standards. In this inspirational role, he also possessed a winning obsessive zeal, mingled with the occasional touch of neurotic punctilio.

Over the years, his group produced countless hundreds of completely new organometallic compounds. Providence had provided, in the form of X-ray crystallography (J. M. Thomas *Nature* 364, 478–482; 1993) the ideal technique to determine their detailed atomic structure, so long as they crystallized satisfactorily — and in his ability to choose the 'right' solvent, Wilkinson had the chemical equivalent of green fingers. An organometallic compound with at least one heavy-metal atom draped with

organic foliage makes X-ray crystal analysis straightforward because it is easy to determine the phases of each X-ray reflection. The structures that he discovered often had unexpected, sometimes bizarre, atomic juxtapositions that challenged conventional valence rules and often perplexed the purist theoretical chemists (whom he relished taunting).

Wilkinson published more than 550 research papers. But he also profoundly influenced the teaching of his subject with *Advanced Inorganic Chemistry*, an undergraduate text which he authored with one of his former American students, F. A. Cotton, in 1962. Adopted in almost every country, and translated into many languages, this text is about to reach its sixth revised edition. The nine-volume encyclopaedic *Comprehensive Organometallic Chemistry* was produced under his leadership in 1982, to be followed in 1995 by a fourteen-volume supplement. In 1983, along with D. C. Bradley, he founded a new inorganic research journal, *Polyhedron*. These ventures alone underline the remarkable pace of discovery in the subject that he had personally done so much to found and advance.

Geoffrey Wilkinson was knighted for his contribution to chemistry in 1976, but he never allowed himself to fall into the 'establishment net'. Indeed his vociferous defence of funding for curiosity-driven research as against a managed 'foresight' programme regularly brought him into conflict with those in authority. He believed strongly, as does Max Perutz, that "in science, wealth-producing discoveries pop up, like Puck, in unexpected corners". He was convinced that too much management of research stifles innovation. Prime ministers, secretaries of state, members of parliament, university vice-chancellors and heads of funding and research councils were among the recurring recipients of his letters. To the very end he prosecuted research, and the Royal Society, who elected him a Fellow in 1965, this year awarded him its prestigious Davy medal.

He is survived by his Danish wife Lise, to whom he was deeply devoted.

John Meurig Thomas & Edward Abel

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Feminization of male carp

SIR — Some common industrial chemicals, previously considered to act only via narcosis (without a specific receptor in an organism)¹, are now suspected to act as endocrine and reproductive disruptors in various species. This conclusion has been reached as a result of *in vitro* screening methods²⁻⁴, molecular probes⁵ and biochemical determinations of vitellogenin in plasma of male fish⁶. Epidemiological studies also suggest a link between exposure to these chemicals with a specific mode of action (for example xeno-oestrogens) and the reproductive disturbances observed in male human and wildlife populations⁷. Here we report the effects of one of these chemicals on male carp.

The effects of industrial chemicals so far detected in feral fish vary from disturbed gonadal maturation and abnormal levels of vitellogenin in adult plasma to intersex gonads (see, for example, ref. 8). Disturbance of the reproductive cycle by chemical insult, principally by endocrine and reproductive disruptors, is likely to affect the dynamics of a species. Particular phases of the reproductive cycle, such as the development of the gonad and male sexual differentiation, are especially sensitive to xeno-oestrogens, as these processes are regulated by endogenous steroid hormones⁹. Natural and synthetic oestrogens are known to induce sex reversal in male fish provided that the treatment takes place during sexual differentiation¹⁰. This has been confirmed for a wide range of fish species¹¹⁻¹³. It is surprising that the use of the sexually undifferentiated stages of fish species has not as yet been explored in aquatic toxicological studies.

We have investigated the effects of exposure to 4-*tert*-pentylphenol (TPP) in male carp during sexual differentiation. Genetically all-male progenies (XY) of the common carp, *Cyprinus carpio*, were obtained by fertilizing eggs of a female

(XX) as described in ref. 14 with sperm from homogametic (YY) males, produced by androgenesis as in ref. 15. Sexually undifferentiated, 50-day-old fish were exposed for 90 days under intermittent

concentrations of some alkylphenolic compounds found in sewage-treatment works¹⁶. We used individuals exposed to 0.01 mg l⁻¹ 17 β -oestradiol (E2) as positive controls and fish exposed only to the dilution water as negative controls.

Six individuals were sampled every 10 days, starting after 30 days of exposure.

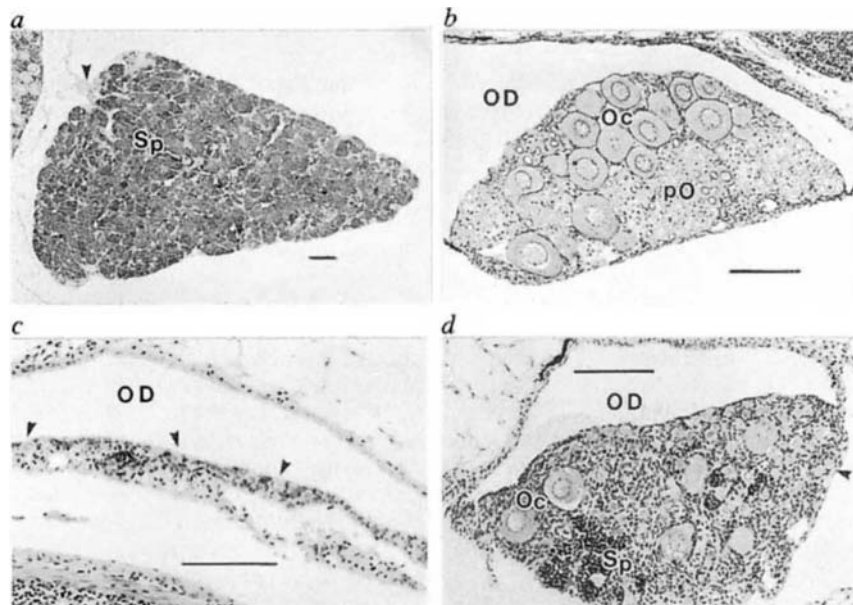


FIG. 2 Gonad of genetic males of *C. carpio*: 4- μ m paraffin cross-sections stained with haematoxylin and eosin. Scale bars, 100 μ m. *a*, Regular testis (XY) from a control male carp. Typically mature male gonad containing all stages of spermatogenic cells. Sp, spermatogenesis; arrow, vas deferens. *b*, Ovary from a normal female carp (XX) presenting the typical oviduct (OD) and oocytes at various stages of maturation: prophase oocytes (pO) and pre-vitellogenic oocytes (Oc). *c*, Poorly developed testis from a genetic male (XY) carp exposed for 60 days to TPP (nominal concentration 1 mg l⁻¹) with an oviduct (OD) and containing only a few PGCs (arrows). *d*, Ovo-testis from a genetic male (XY) carp exposed for 60 days to TPP. Note the simultaneous presence of spermatogenic stadia (Sp) and pre-vitellogenic oocytes (Oc) in this gonad with an oviduct (OD), in addition to primordial germ cells (arrow) and connective tissue.

flow-through conditions to TPP. This period corresponds to the labile period of sex differentiation in the common carp¹³. TPP was chosen as the model compound as it has a potent oestrogenic effect on MCF-7 cells². The range of sublethal nominal concentrations tested is 0.1, 0.32 and 1 mg l⁻¹, which corresponds to the range of

The fish were then anaesthetized and fixed in Bouin's solution, taking a transverse section of each fish at the level of the dorsal fin and embedding it in paraffin to examine the gonad histologically. One of two consecutive sections cut at five levels was stained with haematoxylin and eosin.

We found that exposure for 60 days to all tested TPP concentrations and E2 resulted in the development of an oviduct in almost all male fish. We detected this morphogenetic feature (unique to the female gonad of the common carp¹⁷ and dependent on oestrogens¹⁸) as early as 30 days after the start of exposure to the lowest TPP concentration (0.1 mg l⁻¹; Fig. 1a). Exposure also induced a reduction in the number of primordial germ cells (PGCs) after 40 days in the gonad of fish from all the treatments except the lowest TPP concentration (Fig. 1b).

Figure 2a shows a normal, fully mature XY testis from a control fish after 90 days, characterized by the presence of all spermatogenic stages and a vas deferens. An ovary from a normal XX female is shown in Fig. 2b for comparison. Exposure of the

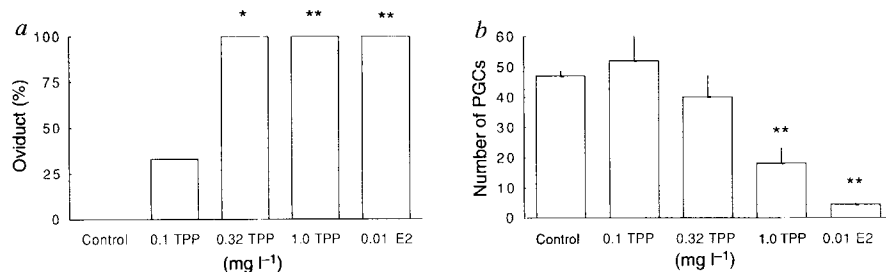


FIG. 1 Effects observed in the gonad of genetic males of the carp exposed for 60 days to TPP and E2 (nominal concentrations in mg l⁻¹). *a*, Frequency of occurrence of an oviduct, observed in cross-sections of gonads from 6–9 individuals per treatment. *, $P < 0.01$; **, $P < 0.001$; χ^2 test. *b*, Total number of primordial germ cells (PGCs) per gonad. PGCs are relatively large cells, round to oval in shape, containing an irregularly shaped nucleus, with one to several nucleoli. Note that the effect is more severe with increasing TPP concentrations. Each value represents the average of the sum of the PGCs counted in both gonads in three sections per fish (3–6 fish per treatment). Bars, standard deviation (only half of the error bars are shown). **, $P < 0.001$; t test.

all-male population to E2 for 90 days resulted in a regular phenotypic female gonad containing an oviduct and oocytes at various stages of development, but no testicular tissue. Exposure to TPP resulted in a very poorly developed testis with an oviduct in most fish where spermatogenesis was severely inhibited (Fig. 2c). In some individuals (2 of 6), exposure to the highest TPP concentration induced the formation of a further feminized testis (ovo-testis, Fig. 2d) and severely inhibited spermatogenesis.

We have demonstrated, for the first time, that the period of sex differentiation in fish is sensitive to xeno-oestrogen compounds, as shown by the appearance of female phenotypic characteristics (oviduct) in the gonad of the genetic male carp. Note, however, that a concentration at which there are no reproductive effects in carp has yet to be established, and will be lower than the nominal concentrations cited here. Nevertheless, we recommend the use of genetically all-male, sexually undifferentiated populations of the common carp for testing *in vivo* the endocrine activity of chemicals and effluents to be discharged to the aquatic environment.

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- Verhaar, H. J. M., van Leeuwen, C. J. & Hermens, J. L. M. *Chemosphere* **4**, 471–491 (1992).
- Soto, A., Lin, T. M., Justicia, H., Silvia, R. M. & Sonnenschein, C. in *Chemically Induced Alterations in Sexual and Functional Development: The Wildlife/Human Connection* (eds Colborn, T. & Clemens, C.) 295–309 (Princeton Scientific, Princeton, 1992).
- Pelissero, C. et al. *J. Steroid Biochem. Mol. Biol.* **44**, 263–272 (1993).
- Jobling, S. & Sumpter, J. P. *Aquat. Toxicol.* **27**, 361–372 (1993).
- Ren, L., Lattier, D. & Lech, J. J. *Bull. Environ. Contam. Toxicol.* **56**, 287–294 (1996).
- Sumpter, J. P. & Jobling, S. *Environ. Health Perspect.* **103** (suppl.), 173–178 (1995).
- Colborn, T., vom Saal, F. S. & Soto, A. M. *Environ. Health Perspect.* **101**, 378–384 (1993).
- Harries, J. E. et al. *Effects of Trace Organisms on Fish — Phase 2* (Foundation for Water Research, Marlow, 1995).
- Thomas, P. in *Casarett and Doull's Toxicology: The Basic Science of Poisons* (eds Amdur, M. O., Doull, J. & Klaassen, C. D.) 484–520 (Pergamon, New York, 1991).
- Yamamoto, T. in *Fish Physiology* (eds Hoar, W. S. & Randall, D. J.) Vol. 3, 117–175 (Academic, New York, 1969).
- van den Hurk, R. & Lambert, J. G. D. in *Proc. Int. Symp. Reprod. Fish.* (eds Goos, H. J. Th. & Richter, C. J. J.) 69–72 (Pudoc, Wageningen, 1982).
- Piferrer, F. & Donaldson, E. M. *Aquaculture* **77**, 251–262 (1989).
- Komen, J. et al. *Aquaculture* **78**, 349–363 (1989).
- Komen, J. et al. *Aquaculture* **92**, 127–142 (1991).
- Bongers, A. B. J. et al. *Aquaculture* **122**, 119–132 (1994).
- Naylor, C. G. *Soap Cosmet. Chem. Special* **68**, 27–32 (1992).
- Komen, J., Yamashita, M. & Nagahama, Y. *Dev. Growth Differ.* **34**, 535–544 (1992).
- Komen, J., Lambert, J. G. D., Richter, C. J. J. & Goos, H. J. Th. in *Proc. Fifth Int. Symp. Reprod. Endocrinol. Fish* (eds Goetz, F. W. & Thomas, P.) 383 (Univ. Texas at Austin, 1995).

Recovery of Antarctic ozone hole

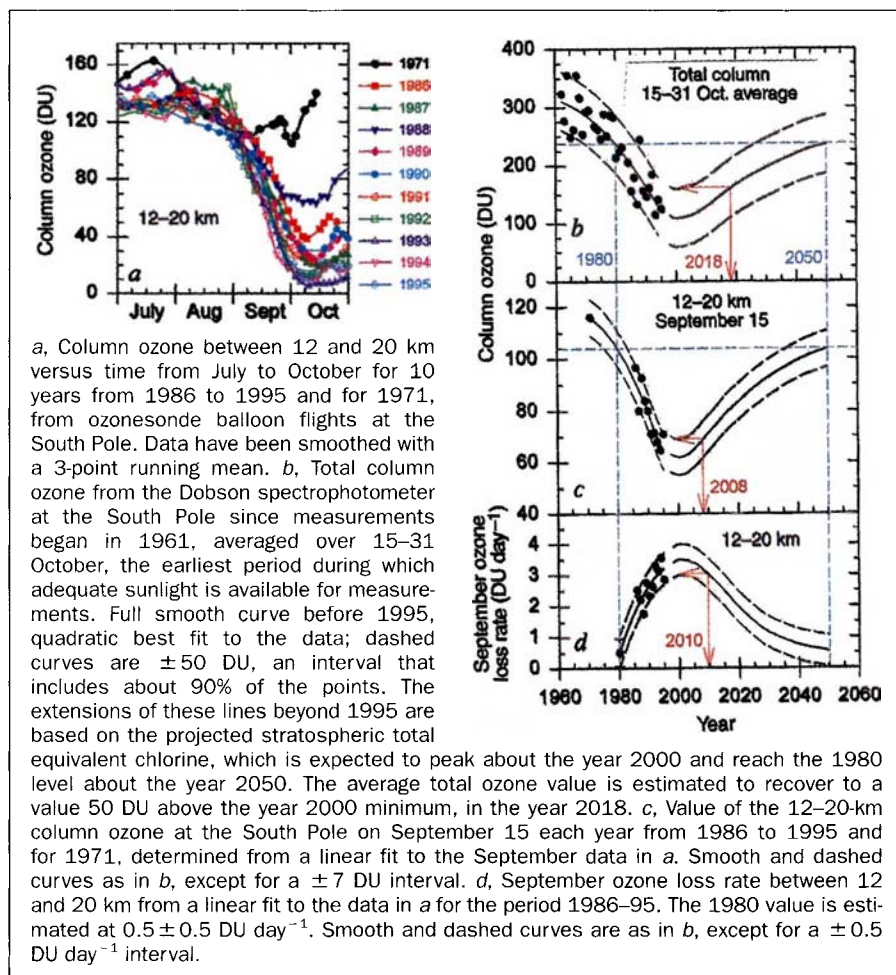
SIR — A ban on production of various chlorofluorocarbons (CFCs) and halons began in January 1996, as required by the Montreal Protocol on substances that deplete the ozone layer and subsequent amendments¹. Continuous global monitoring now indicates that the total ozone-destroying potential of the atmosphere (in terms of total equivalent chlorine using an amplification factor of 40 for the bromine in halons) peaked in the troposphere in 1994 and, barring future non-compliance with the Montreal Protocol, will peak in the stratosphere between 1997 and 1999 (ref. 2). The ozone layer would then begin to heal slowly, reaching pre-ozone-hole levels by about the year 2050, when stratospheric equivalent-chlorine will reach a mole fraction of about 2 parts per billion (p.p.b.)¹, a level believed to have been present when the Antarctic ozone hole clearly emerged in 1980 (ref. 3).

It is likely that the first signs of healing of the ozone layer will be observed over Antarctica simply because the springtime depletion there is very large in comparison to natural variability, whereas the mid-latitude depletions are of the same order of magnitude as the variability. Detection of the first signs of healing of

the ozone hole is important as it will provide verification of the research that provided the justification for the banning of certain ozone-depleting substances. It is possible that this detection will occur early in the twenty-first century.

Total ozone measurements made from the ground at the Amundsen–Scott Station at the South Pole for the 15–31 October period since 1961 show an interannual variability of about ± 50 Dobson units (DU; *b* in the figure) or about 50% of the minimum value expected at about the year 2000. This variability is partially related to polar-vortex disturbances which begin to appear in late October in some years. Thus, it is unlikely that the beginning of recovery of springtime total ozone as measured on the ground will be detected at the South Pole within the next 20 years, based on predicted¹ stratospheric equivalent-chlorine levels (*b*).

For 10 years, year-round ozone balloon-soundings have been conducted at the South Pole and analysed in a search for parameters that might allow earlier detection of the beginning of recovery of the ozone hole. These measurements can be used to obtain ozone in the altitude range from 12 to 20 km (*a* in the figure),



where 80–90% of the ozone is lost each spring (September and early October). In the absence of temperature and volcanic aerosol variations, which affect heterogeneous chemical ozone depletion^{4,5}, the rate of ozone loss in September (d in the figure) should vary solely with halogen levels. However, this rate has an inter-annual variability of about ± 5 DU day⁻¹, or 15% of the maximum value expected in about 2000. This variability may be related to transport variations associated with the quasi-biennial oscillation (QBO)⁶. It may also be related to the polar vortex being displaced from the Pole, which can result in South Pole air parcels being subjected to sunlight and enhanced photochemistry earlier than normal.

The amount of ozone remaining in the 12–20-km altitude interval on 15 September (S15) has shown a consistent downward trend during the past 10 years (c in the figure), with a variability of roughly 7 DU, or 10% of the minimum value expected in about 2000. The smaller variabilities of S15 and the ozone-loss rate are probably related to the relative stability of the vortex during the mid-September period. A comparison with balloon measurements made at the South Pole in 1971 (ref. 7) is shown in the figure, indicating that the beginning of the reduction in S15 is consistent with that of total ozone, possibly as early as 1970. The latter possibility was also identified in the daily October minimum total ozone at Halley, Antarctica⁸.

Owing to their lower variability, the ozone-loss rate and S15 are likely to be suitable early ozone-healing indicators. Through continued monitoring of the ozone profile at the South Pole, it is estimated that detection of the turnaround should be possible by the year 2008, about 10 years earlier than seems possible by monitoring total ozone alone. This estimate obviously assumes that the downward trend in the total equivalent-chlorine content of the atmosphere continues. If there were a large volcanic eruption in future, for example, the resultant aerosol particles would augment polar stratospheric cloud-related heterogeneous chemistry and affect Antarctic ozone in the lower stratosphere⁵. But these particles would probably not increase ozone loss variability more than the Pinatubo eruption in 1992 and 1993. In addition, future downward stratospheric temperature trends during winter and spring, as have occurred over Halley only in October and November³, would increase the occurrence

of polar stratospheric clouds and prolong recovery. Even so, this is not expected to be a major factor in the next 10 years.

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1. Scientific Assessment of Ozone Depletion: 1994 World Meteorological Organization, Global Ozone Research and Monitoring Project Report No. 37 (Geneva, 1995).
2. Montzka, S. A. et al. *Science* **272**, 1318–1322 (1996).
3. Farman, J. C., Gardiner, B. G. & Shanklin, J. D. *Nature* **315**, 207–210 (1985).
4. Solomon, S. *Nature* **347**, 347–354 (1990).
5. Hofmann, D. J. et al. *Geophys. Res. Lett.* **22**, 2493–2496 (1995).
6. Hofmann, D. J. *Nature* **382**, 129 (1996).
7. Oltmans, S. J., Hofmann, D. J., Kornhyr, W. D. & Lathrop, J. A. in *Proc. Quadrennial Ozone Symp. 1992* NASA CP-3266, 578–581 (1994).
8. Jones, A. E. & Shanklin, J. D. *Nature* **376**, 409–411 (1995).

Genes from wild rice improve yield

SIR — Although the wild relatives of crop species are often inferior to modern cultivars, recent evidence suggests that they may contain genes capable of improving both the yield and quality of modern varieties¹. However, these favourable genes are often masked by the effects of other deleterious genes. Molecular-genetic maps can be used to exploit the genetic potential of wild species for the improvement of yield and quality in modern plant cultivars².

We have tested this strategy in rice by screening a single accession of the wild species, *Oryza rufipogon*, a weedy relative of cultivated rice, for genes capable of increasing the yield of one of China's highest-yielding cultivars. *O. rufipogon*, like other wild relatives of rice, has never before been exploited for the genetic improvement of rice yields.

The world's annual rice production will have to increase 70% by the year 2030 to keep up with the demands of a growing population³. In the past, increases in production of this staple food were largely achieved by expanding the area under cultivation, increasing fertilizer inputs and using chemical pest control, but none

of these options is realistic today. Future productivity increases will require raising the genetic yield potential of the species, but the yield potential of modern rice varieties has remained stationary for many years.

V/64 is one of the top-performing F₁ hybrid rice varieties in China and is derived from a cross between the two inbred lines V20A and Ce64. We used a Malaysian accession of *O. rufipogon* (IRGC 105491) as the male in a cross to V20A, a cytoplasmic male sterile line, to produce an interspecific F₁ hybrid which expressed strong vegetative vigour. We crossed the F₁ with V20B (maintainer line of V20A, having the same nuclear genome as V20A) to generate 52 BC₁ plants which were grown in the field in the summer of 1993.

The best 10 BC₁ plants, selected for desirable plant type, maturity and fertility, were backcrossed a second time to V20B to generate more than 3,000 BC₂ plants. From these, we selected a subset of 300 and crossed them with Ce64 to generate 300 BC₂ test-cross families. Each of these families should have the genetic constitution of the original V/64 hybrid variety, with the exception of small substitutions of chromosome segments from *O. rufipogon* (on average, each BC₂ test-cross line contained 5% *O. rufipogon*-derived DNA).

We evaluated these BC₂ test-cross families, together with the V/64 hybrid, V20B and *O. rufipogon* parents, for yield in summer 1994 at the China National Hybrid Rice Research and Development Center, where most of China's hybrid rice varieties are developed. We planted three-row plots with 11 plants per row in a randomized complete block design with two replications, and evaluated grain yield per plot.

The figure shows the distribution of grain yield observed for the BC₂ test-cross families and the parental controls. *O. rufipogon* plots were among the lowest performers, yielding 55% less than the V/64 hybrid control and possessing fewer grains per plant and a smaller 1,000-grain weight. This was expected given that *O. rufipogon* is not a cultivated species.

Most of the BC₂ test-cross families also

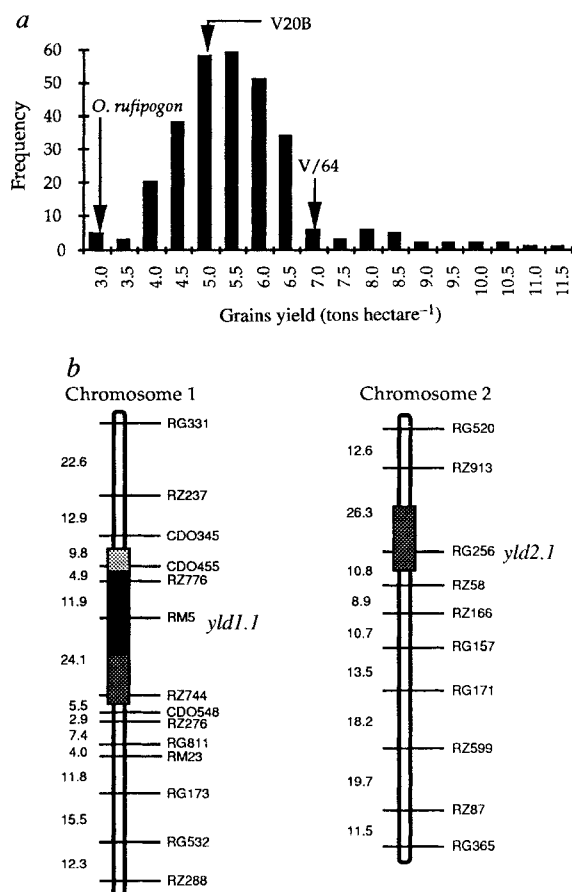
CHARACTERISTICS OF POSITIVE WILD QTL ALLELES ASSOCIATED WITH YIELD

QTL	Marker	Chr	P	V/C class		(1/2 V/C + 1/2 R/C) class		R/C class	Allele effect	% of V/64
				PM	N	PM	N	PM		
yld1.1	RM5	1	0.0036	5.77	251	6.38	45	6.99	1.22	18.26
yld2.1	RG256	2	0.0058	5.76	247	6.33	49	6.90	1.14	17.07

V/C and R/C refer to genotypic classes defined by single markers: V/C is the heterozygote (V20A/Ce64) and R/C the interspecific heterozygote (*O. rufipogon*/Ce64) in the BC₂ test-cross population. PM is the phenotypic mean of a class. The PM of R/C is 2 × PM of (1/2 V/C + 1/2 R/C) – PM of V/C, assuming regular segregation within families. P is the probability that the marker genotype was not associated with yield. N, number of families within a genotypic class; Chr, chromosome number.

Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. Priority will be given to letters of fewer than 500 words and ten references.



a, Frequency distribution of yield in an interspecific (*O. sativa*/*O. rufipogon*) BC₂ test-cross population, with phenotypes of *O. rufipogon*, V20B and V/64 (V20A×Ce64) indicated by arrows; b, chromosome maps (marker order and map distances based on published rice molecular-genetic map⁴) showing locations of putative yield-enhancing genes from *O. rufipogon*, *yld1.1* and *yld2.1*. *O. rufipogon* allele associated with increased yield at $P < 0.005$ (black shading); $P < 0.010$ (dark grey shading); and $P < 0.050$ (light grey shading).

yielded less than V/64, but a small percentage of the plots yielded as much as 50% greater than the elite hybrid. Overall, 15% of the BC₂ test-cross families outperformed V/64 with respect to yield, 14% with respect to grains per plant, and 56% with respect to 1,000-grain weight. Thirteen (4.3%) of the BC₂ test-cross families outyielded V/64 by at least 30%. These results suggest that genes coming from *O. rufipogon* can increase yield of an elite rice variety, even though *O. rufipogon* itself is inferior to cultivated rice varieties.

If *O. rufipogon* genes are contributing to the higher yield of certain BC₂ test-cross families, it should be possible to detect the presence of the segments of wild chromosomes containing such genes (quantitative trait loci or QTL) and to demonstrate a significant correlation between their presence and higher yield.

A high-density molecular-genetic map developed for rice⁴ has previously been used to map and characterize QTL conditioning heterosis⁵, biotic and abiotic stress

tolerance and various agro-nomic traits^{6,7}. In an effort to identify regions of the *O. rufipogon* genome that might be contributing to a yield increase, 100 informative restriction-fragment length polymorphisms and 20 microsatellite markers (S. R. McC. *et al.*, unpublished data), covering the entire rice genome at intervals of roughly 12 cM, were assayed on each of the 300 BC₂ test-cross families.

We conducted QTL mapping on BC₂ test-cross data by regression of field performance on marker genotype using standard analysis of variance procedures and assuming regular segregation of wild and cultivated alleles within test-cross families. In most cases, introgression of *O. rufipogon* alleles had either no significant effect on yield or were inferior to the cultivated alleles. However, *O. rufipogon* alleles at marker loci RM5 on chromosome 1 and RG256 on chromosome 2 were associated with enhanced yield ($P < 0.006$; see table on previous page), and were designated *yld1.1* and *yld2.1* (see figure). The phenotypic advantage of the lines carrying *O. rufipogon* alleles at these loci was estimated to be 1.2 and 1.1 tonnes per hectare (fig-

ures converted to field scale using conventional plant densities), respectively, which corresponds to an 18 and 17% increase over V/64 (see table).

The alleles *yld1.1* and *yld2.1* were both associated with a significant increase in grains per plant ($P < 0.005$), but had no detectable effect on 1,000-grain weight, plant height or growth duration.

Rice was originally domesticated by humans from wild stands of native plants⁸. Only a small portion of the genetic variation found in nature was captured in this domestication process⁹, and it is this limited genetic base that forms the foundation of all of our modern cultivars¹⁰. Many of the ancestors of cultivated crop species still exist in the wild or have been collected and maintained in germplasm banks. Although these wild species are valued as a unique source of genetic variation, they usually have low yields. Nevertheless, our results indicate that one of the closest wild relatives of cultivated rice, *O. rufipogon*, despite its overall inferior appearance, contains

genes that can substantially increase the yield of rice. The strategy we describe here is being used to search for yield-enhancing genes in other wild rice species, and could be extended to the genetic improvement of other crop species.

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1. Tanksley, S. D. *et al.* *Theor. Appl. Genet.* **92**, 213–224 (1996).
2. Tanksley, S. D. & Nelson, J. C. *Theor. Appl. Genet.* **92**, 191–203 (1996).
3. IRRI 1993–1995 *IRRI Rice Almanac* (Manila, Philippines, 1993).
4. Causse, M. A. *et al.* *Genetics* **138**, 1251–1274 (1994).
5. Xiao, J., Li, J., Yuan, L. & Tanksley, S. D. *Genetics* **140**, 745–754 (1995).
6. McCouch, S. R. & Doerge, R. W. *Trends Genet.* **11**, 482–487 (1995).
7. Xu, Y. *et al.* *Sci. China* **38**, 422–428 (1995).
8. Oka, H. I. *Origin of Cultivated Rice* (Japan Sci. Soc., 1988).
9. Wang, Z. Y., Second, G. & Tanksley, S. D. *Theor. Appl. Genet.* **83**, 565–581 (1992).
10. Ladizinsky, G. *Econ. Bot.* **39**, 191–199 (1985).

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Origin of oscillons

SIR — The term ‘oscillons’, recently described in *Nature*^{1,2} and featured on the cover, was earlier used by us³ to represent strongly nonlinear electrostatic oscillations on a plasma boundary. These peculiar oscillations of surface fields are described by exact solutions of the cold electron fluid and Maxwell equations together with the boundary conditions. They have a unique spatial pattern and their frequency satisfies a simple algebraic dispersion relation involving the oscillon radius and peak amplitude.

Clearly, these oscillons are physically different from those recently discussed in *Nature*. But in all cases, strongly nonlinear anomalous oscillations of surface patterns are involved, and thus there is no fundamental conflict in the terminology.

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1. Fineberg, J. *Nature* **382**, 763–764 (1996).
2. Umbanhowar, P. B., Melo, F. & Swinney, H. L. *Nature* **382**, 793–796 (1996).
3. Stenflo, L. & Yu, M. Y. *Phys. Fluids B* **1**, 1543–1544 (1989).

An essential bipolar mitotic motor

SIR — Separation of the mitotic spindle at the poles during cell division depends on the action of members of the bimC family of proteins. These proteins are part of the kinesin superfamily of 'motor' proteins; molecules that bind microtubules and produce mechanical work from chemical energy¹. The mechanism of action of bimC motors is not yet known. We report here that the bimC-related *KLP61F* gene, an essential mitotic gene of the fruitfly *Drosophila melanogaster*, encodes subunits of the bipolar kinesin, KRP₁₃₀, a homotetrameric complex of four motor subunits, each of relative molecular mass 130,000 (*M_r* 130K), assembled into a bipolar 'minifilament'². This result suggests that bipolar bimC motors work by crosslinking and sliding apart antiparallel microtubules, causing the separation of duplicated spindle poles and allowing bipolar spindle assembly.

To identify the gene encoding the KRP₁₃₀ subunit, we performed protein microsequencing of purified *Drosophila* embryonic KRP₁₃₀ (refs 2, 3). Proteolysis of KRP₁₃₀ yielded tryptic peptides, which we fractionated, and we selected the seven best-resolved fractions for further analysis.

Map based on the amino-acid sequence of *Drosophila* KLP61F (ref. 5), indicating positions (grey boxes) and amino-acid sequences of KRP₁₃₀-derived peptides (top rows of sequences), corresponding regions of KLP61F (middle rows), and bimC family consensus sequence (bottom rows). Numbers above indicate amino-acid residues bordering the motor (residues 1–354), stalk (354–960) and tail (960–1,066). Single-letter amino-acid code; X indicates unresolved residues. The indicated peptide sequences correspond to residues 123–136, 231–242, 257–270, 339–352, 391–402, 492–516, 542–552, 669–685 and 880–896 for KLP61F. We found 100% identity between KRP₁₃₀ and KLP61F sequences, but little or no homology with the bimC family consensus sequence. Residues highlighted in red are identical in all bimC motors. We immobilized sucrose gradient-purified KRP₁₃₀ on membranes and performed *in situ* proteolysis with trypsin⁷. We analysed selected peptides by matrix-assisted laser desorption-ionization time-

of-flight mass analysis, using α -cyano-4-hydroxycinnamic acid as the matrix. The nine unique peptides thus identified matched the predicted masses of KLP61F tryptic fragments (using the MSFIT program at the UCSF mass spectrometry facility to search *Drosophila melanogaster* proteins in the SwissProt.r33 database). These peptides were analysed by automated Edman degradation, yielding reliable sequences (illustrated). bimC consensus sequences in the indicated regions were determined by lineup comparison of *Drosophila* KLP61F, *Xenopus* Eg5, human HsEg5, *Aspergillus* bimC, *Saccharomyces pombe* Cut7 and *S. cerevisiae* CIN8 and KIP1. Full methodological details available on request from J. M. S.

One of these fractions contained a 17-residue peptide, whose sequence we determined. A database search revealed a 100% match of this peptide with a segment of the nonconserved stalk of a previously identified *Drosophila* bimC-related protein, KLP61F (see figure). Further analysis of the peptide fractions revealed that they contain a total of nine peptides whose masses match exactly those of the predicted tryptic fragments derived from the *Drosophila* KLP61F protein sequence^{4,5}. To confirm that KRP₁₃₀ is identical to KLP61F, we sequenced all nine of these peptides and found that they display 100% identity with the corresponding segments of the deduced KLP61F protein sequence, but differ in many positions from the bimC family consensus sequence (figure). We therefore conclude that a KLP61F polypeptide is identical to a subunit of the bipolar kinesin, KRP₁₃₀.

This discovery contradicts the hypothesis that KLP61F and KRP₁₃₀ differ⁶, and bridges the gap between genetic evidence concerning the biological function of KLP61F (ref. 5), studies of its expression and localization on spindle micro-

tubules^{5,6}, and biochemical and electron-microscope studies of the recombinant polypeptide⁶ and the native bipolar holoenzyme^{2,3}. For example, in *Drosophila* KLP61F mutants, the absence of KLP61F function leads to the formation of monopolar spindles with unseparated spindle poles³. This observation, together with the results described here, indicates that the essential mitotic KLP61F gene might encode a slow plus-end-directed microtubule motor polypeptide^{3,6} that self-assembles into a bipolar homotetrameric holoenzyme² capable of crosslinking and sliding apart antiparallel microtubules, thereby pushing apart the associated spindle poles during spindle assembly and function.

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1. Walczak, C. E. & Mitchison, T. J. *Cell* **85**, 943–946 (1996).

2. Kashina, A. S. *et al.* *Nature* **379**, 270–272 (1996).

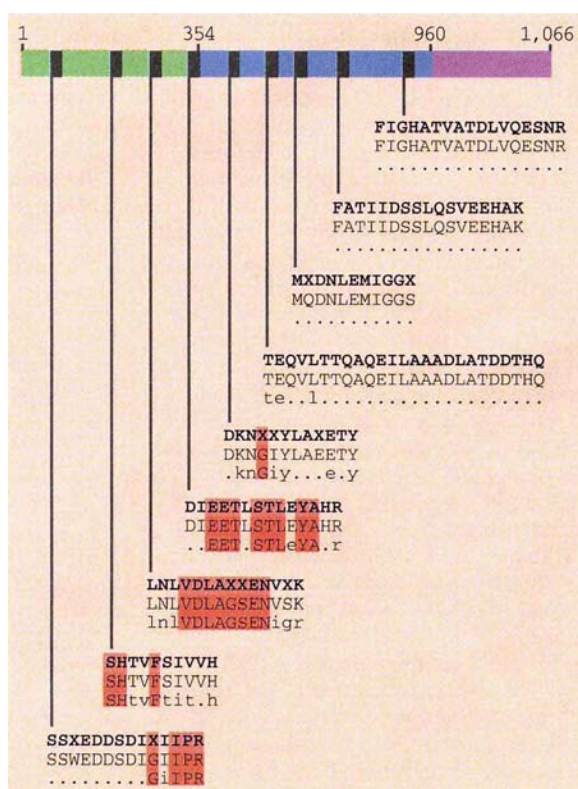
3. Cole, D. G., Saxton, W. M., Sheehan, K. B. & Scholey, J. M. *J. Biol. Chem.* **269**, 22913–22916 (1994).

4. Stewart, R. J., Pesavento, P. A., Woerpel, D. N. & Goldstein, L. S. B. *Proc. Natl Acad. Sci. USA* **88**, 8470–8474 (1991).

5. Heck, M. S. *et al.* *J. Cell Biol.* **123**, 665–679 (1993).

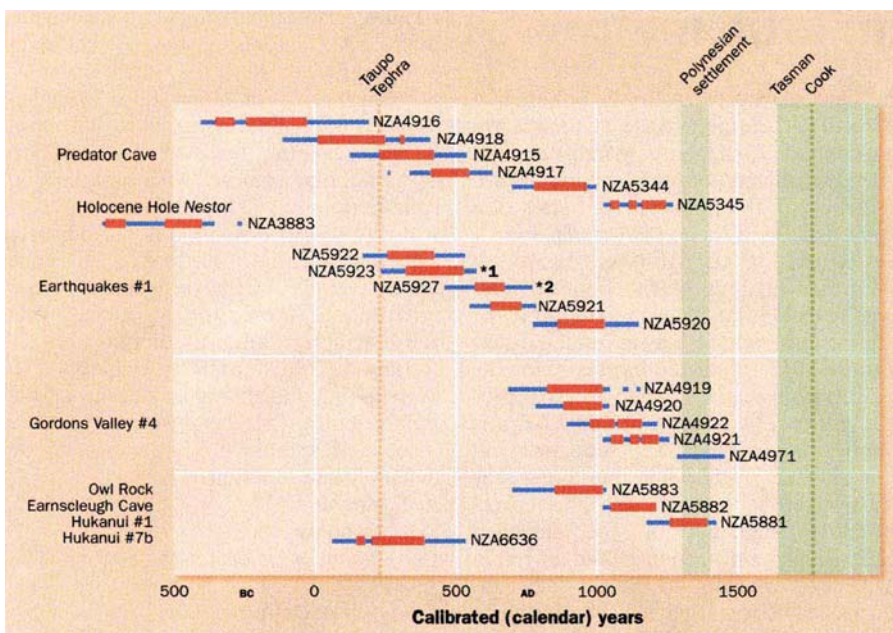
6. Barton, N. R., Pereira, A. & Goldstein, L. S. B. *Mol. Biol. Cell* **6**, 1563–1574 (1995).

7. Fernandez, J., Andrews, L. & Mische, S. M. *Anal. Biochem.* **218**, 112–117 (1994).



Arrival of rats in New Zealand

SIR — The assumption that the commensal Pacific rat, *Rattus exulans*, arrived in New Zealand at the time of human settlement¹ has never been tested. Unequivocal evidence for human settlement in New Zealand dates from no earlier than about 850 years before present (yr BP)^{2,3}, although the date of colonization is still controversial^{2,4,6}. I report radiocarbon ages of up to about 2,000 yr BP on bone gelatin from Pacific rats from both main islands of New Zealand that imply an early, transient, human contact with New Zealand more than 1,000 years before settlement. Extinctions of small vertebrates vulnerable to rat predation should precede extinctions of moa and other large taxa by human hunting and habitat destruction.



Radiocarbon ages of *Rattus exulans* specimens from North and South Islands, New Zealand and comparative ages for other taxa, expressed as calibrated (calendar) years^{15–19}. Blue lines, 95% confidence interval(s); red lines, 68% confidence interval(s). Narrow green band indicates calibrated ages for 70% of radiocarbon dates from human sites in the South Island². Wide green band, period of European influence; “Tasman” and “Cook” indicate dates of initial contacts by European explorers. “Holocene Hole Nestor”, calibrated bone gelatin age on South Island kaka parrot *Nestor meridionalis* (herbivore) from a small-vertebrate site without the Pacific rat¹¹, gives maximum age for the presence of the Pacific rat in the South Island. *1, NZA5923, on New Zealand pigeon *Hemiphysa novaeseelandiae* gelatin; *2, NZA5927, on South Island kokako *Callaeas cinerea* gelatin; both herbivores.

Humans have transported the Pacific rat throughout the central and South Pacific¹ from its presumed area of origin in southeast Asia. New Zealand is at the southern extremity of the range of both the Pacific rat and humans^{7,8}. The earliest evidence of permanent human occupation and palynological evidence for major environmental disturbance in New Zealand both date from 800–850 radiocarbon yr BP (ref. 3). Humans and Pacific rats have been implicated in the extinctions that affected the vertebrate and invertebrate faunas of New Zealand^{9,10}, but their relative effects have been difficult to assess⁹.

I obtained 18 accelerator mass spectrometry (AMS) ¹⁴C dates on the gelatin fraction from Pacific rat bones collected from eight natural, mostly predator-accumulated, deposits on the North and South Islands (see figure). Supplementary information on samples and treatments is available from R. N. H. and is on the *Nature* Web site (<http://www.nature.com>). The older date from the North Island (NZA6636, 1,775 ± 93 yr BP) is slightly younger than the oldest from the South Island (NZA4916, 2,155 ± 130 yr BP; NZA4918, 1,930 ± 110 yr BP), but the difference in calibrated dates is not significant (see figure). Earlier dates are unlikely, at least in the South Island; a site rich in small vertebrates, but lacking rats, had a ¹⁴C date span of 2,955 ± 79 (NZA3813) to

2,423 ± 65 (NZA3883) yr BP (ref. 11).

The results show that 15 Pacific rat dates (see figure) are significantly older than the generally accepted date for permanent human settlement^{2,3}. Therefore I did tests to see if the AMS gelatin dates were biased by unknown sources of contamination, as has been suggested for ‘collagen’ dates². Potential sources of error include the addition of ‘old’ or reservoir carbon to the bone gelatin before death in the diet, or after deposition via unremoved humics or diagenetic processes in carbonate sedimentary environments, especially for small specimens.

Dietary influences were not apparent. Two individuals of known death date give calibrated ages (NZA5876, 6087) that include their death dates. In addition, ¹⁴C dates (NZA5923, NZA5927) on bone gelatin from two herbivorous birds (equilibrium carbon consumers) are not significantly different from those on rat bones from comparable levels (NZA5922 and NZA5921, respectively; see figure). Humic contamination is unlikely, most being removed by gelatinization¹², but must still be considered for earlier ‘collagen’ dates. Environmental carbonates were removed by an acid pre-wash¹², eliminating carbonate contamination¹³. Measured ages were not related to whole-sample mass.

Longer-term diagenetic changes do not appear to have a significant effect. Samples of moa eggshell (species unknown)

(NZA6627) and bird bone (NZA6190) from close proximity in sediment enclosed by two undisturbed volcanic tephra (Taupo Tephra, 1,850 ± 10 yr BP; Waimihia Lapilli, 3,280 ± 20 yr BP; ref. 14) give indistinguishable ages (2,905 ± 88 and 2,995 ± 72 yr BP, respectively). These materials were prepared using different treatments. Finally, a rat dentary excavated from beneath the Taupo Tephra gives an age of 1,775 ± 93 yr BP (NZA6636). In addition to the radiocarbon age being consistent with that of the covering tephra, the bone’s position beneath the undisturbed layer provides independent evidence that Pacific rats were established in the North Island before the Taupo eruption.

The data suggest that the Pacific rat was established on both main islands of New Zealand nearly 2,000 years ago. The rat is unlikely to have arrived without human assistance, on, for example, natural rafts¹. It is more likely that rats were introduced by transient human visitors, who either left immediately or quickly died out. For more than 1,000 years after its arrival, the Pacific rat was the sole exotic predator in New Zealand. Examination of the fossil record should show that small vertebrates and large invertebrates susceptible to rat predation became extinct before larger taxa were eliminated by human hunting and habitat destruction. The hiatus between the introduction of Pacific rats and human colonization means that the faunal extinction in New Zealand had at least two stages.

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1. Roberts, M. *Pacif. Sci.* **45**, 123–130 (1991).
2. Anderson, A. J. *Antiquity* **65**, 767–795 (1991).
3. McGlone, M. S., Anderson, A. J. & Holdaway, R. N. in *The Origins of the First New Zealanders* (ed. Sutton, D. G.) 136–163 (Auckland Univ. Press, 1994).
4. Anderson, A. J. *J. Polynesian Soc.* **104**, 110–132 (1995).
5. Sutton, D. G. *NZ J. Archaeol.* **9**, 135–155 (1987).
6. Kirch, P. V. & Ellison, J. *Antiquity* **68**, 310–321 (1994).
7. Matissou-Smith, E. J. *Polynesian Soc.* **103**, 75–87 (1994).
8. Wodzicki, K. & Taylor, R. H. *Acta Zool.* **172**, 99–101 (1984).
9. Holdaway, R. N. *NZ J. Ecol.* **12** (suppl.), 11–25 (1989).
10. Whitaker, A. H. *Proc. NZ Ecol. Soc.* **20**, 121–130 (1973).
11. Worthy, T. H. & Holdaway, R. N. *J. R. Soc. NZ* **25**, 333–370 (1995).
12. Redvers-Newton, N. & Coote, G. E. *Nucl. Instrum. Methods Phys. Res. B* **92**, 270–273 (1994).
13. Marcus, L. F. & Berger, R. in *Quaternary Extinctions: A Prehistoric Revolution* (eds Martin, P. S. & Klein, R. G.) 159–183 (Univ. Arizona Press, Tucson, 1984).
14. Froggatt, P. C. & Lowe, D. J. *NZ J. Geol. Geophys.* **33**, 89–109 (1990).
15. Bard, E., Arnold, M., Fairbanks, R. G. & Hamelin, B. *Radiocarbon* **35**, 191–199 (1993).
16. Kromer, B. & Becker, B. *Radiocarbon* **35**, 125–135 (1993).
17. Linick, T. W., Long, A., Damon, P. E. & Ferguson, C. W. *Radiocarbon* **28**, 943–945 (1986).
18. Pearson, G. W. & Stuiver, M. *Radiocarbon* **35**, 25–33 (1993).
19. Stuiver, M. & Pearson, G. W. *Radiocarbon* **35**, 1–23 (1993).

Past views of the planets

Stuart Ross Taylor

A History of Modern Planetary Physics. Three volumes*. By Stephen G. Brush. Cambridge University Press: 1996. Pp. 800. £100, \$145.

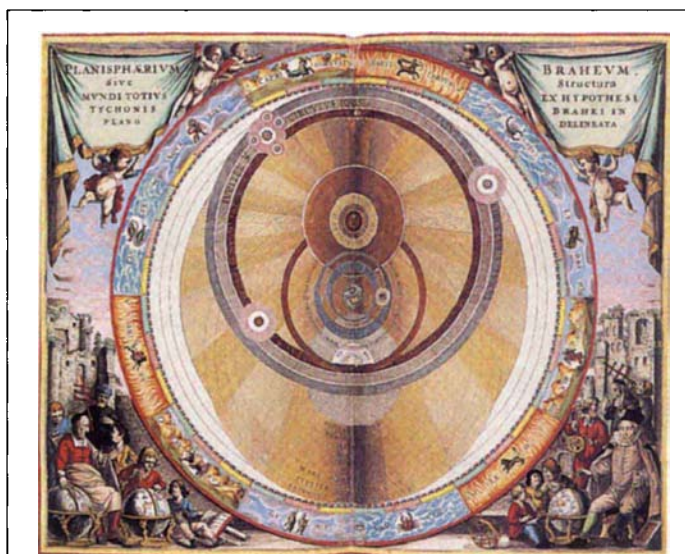
STEPHEN Brush is a physicist turned historian of science. Much of his work on the history of planetary physics has appeared in scientific journals of limited circulation, and so has mostly been known only to specialists. Now he has brought his studies together in a useful three-volume set. The connecting thread running through them is the question of the origin of the Solar System. The subject is of such broad interest and written about with such insight that this book should be read by scientists in all disciplines.

Unlike many workers in the history of science, Brush writes with the advantage of inside knowledge and concludes that "it is reasonable to compare styles of research on the history of the Earth with styles of research on the history of human society". He draws an amusing contrast between the working habits of historians and scientists, writing with the perception of someone who has had a foot in each camp: "The most striking difference between historians and geologists... is that the latter are constantly interacting with each other whereas the former seem to work in isolation."

He reminds us that planetary science once occupied the attention of scientists of the stature of Dalton, Gauss and Kelvin, yet by the end of the nineteenth century it had lost its position among the 'pure' sciences and had become an occupation "suited only to second-raters". Certainly, planetary science, heavy on descriptive material, has had a long struggle to regain its position. Part of the problem was that "geology was moribund... from about 1860 to 1940 because it lacked the techniques needed to solve its important problems" and "geologists in the twentieth century became accustomed to carrying on interminable controversies about problems that they were unable to solve".

Volume One begins with a lengthy dis-

cussion of attempts to understand the Solar System, from the initial work of Pierre Simon, Marquis de Laplace, in 1796. Newton had assumed that the world was created essentially in its present form. God had ordered each planet to move in its particular orbit. How otherwise could one explain the regularities in the system?



A 1660 version of the Danish astronomer Tycho Brahe's planetary system incorporating Galileo's discovery of the four moons of Jupiter. Tycho is seated bottom right, measuring a globe. From *The Cambridge Illustrated History of Astronomy* edited by Michael Hoskin. Cambridge University Press, £24.95, \$39.95.

Laplace, however, was an inhabitant of the Age of Enlightenment and had a more rational explanation. Brush repeats the story about how Laplace gave a copy of his book to Napoleon. Seeing no mention of God, presumably the designer of the system, Bonaparte asked Laplace about the omission. Laplace made his famous reply that he had "no need for that hypothesis".

Brush frequently steps aside to consider other aspects of the history of planetary physics, and in the next section he sidetracks to consider the discovery of, and explanations for, the core of the Earth.

Volume Two begins with a discussion of geology as a science. Brush covers the debate between Kelvin and the geologists about attempts to estimate the age of the Earth. Curiously, following the discovery of radioactivity and so having won the battle with Kelvin, geologists were reluctant

to accept the great periods of time that Lyell had demanded and that were now revealed by the discovery of rocks and minerals that were billions of years old. Geological versions of the time taken to deposit strata and to accumulate the sodium content in the oceans gave estimates of a few hundred million years at most. Chamberlin emerges as the hero of the debate: using a combination of geological and biological assumptions, he estimated the age of the Earth at 4.26 billion years.

Brush turns to the conflict between terrestrial and cosmological timescales in the 1940s. By that time, an age for the Earth in excess of 3 billion years was well established. Astronomical estimates based on the Hubble parameter converged at around 2 billion years. So the Earth was older than the Universe. But revision of the Hubble constant remedied this anomaly, and made the important contribution of detaching the problem of the origin of the Solar System from that of the Universe at large. More recently, we have recognized that planet formation is a contemporary process, not something that occurred long ago. This point has been made more dramatically by discoveries of other planetary systems, none of which, incidentally, seems to resemble our own.

Alfred Nier emerges as another hero, thanks to his construction of mass spectrometers and his measurement of the relative abundances of the lead isotopes, opening the way for establishing the age of the Earth. This was accomplished by Clair Patterson whose value of $4,550 \pm 70$ million years (here one of the book's few typographical errors occurs) has stood for more than 40 years — all subsequent workers have found "a result lying within the original limits of error".

Volume Two concludes with a short section on the origin of the elements. Although credit for this work mostly goes to the famous B²FH group of Fowler, Hoyle and the Burbidges, Al Cameron was also one of the workers who established the present consensus on element synthesis, a fact known only to specialists. Nier's work before the Second World War on isotopic abundances was again a crucial first step.

In Volume Three, the author returns to his main thesis, the history of attempts to understand the Solar System. During the last century, "cosmogony was not quite a

*Vol. 1: Nebulous Earth — The Origin of the Solar System and the Core of the Earth from Laplace to Jeffreys. Vol. 2: Transmuted Past — The Age of the Earth and the Evolution of the Elements from Lyell to Patterson. Vol. 3: Fruitful Encounters — The Origin of the Solar System and of the Moon from Chamberlin to Apollo.

respectable subject for [astronomers]. Instead, dispute about the origin of the Solar System was left to amateurs." In this century it became slightly more respectable, moving, as Brush remarks, "from underground to underdog". At the beginning of his discussion of modern (post-1956) theories, he comments that "attempts to find a plausible naturalistic explanation of the origin of the Solar System began about 350 years ago (with Descartes) but have not yet been quantitatively successful, making this one of the oldest unsolved problems in modern science".

There is an entertaining discussion that begins with the planetesimal hypothesis of Chamberlin and Moulton and concludes with the giant impact hypothesis for the origin of the Moon. The argument between monistic and dualistic theories seems to have been resolved in favour of the former, although Brush warns us about the wide swings of opinion in the past. However, the widespread evidence for dusty discs around stars and the discovery of new "planets, brown dwarfs", or whatever these new objects are, seems finally to have confirmed monistic theories for the formation of planetary systems.

Problems with the nebular hypothesis late last century led to the tidal hypotheses of Chamberlin and Moulton, and those of Jean and Jeffreys. These in turn were mostly discredited by the middle of this century. Among fatal objections were that filaments of material drawn from the Sun will not condense and that the planets contain too much lithium, beryllium and boron (which are destroyed in nuclear reactions in the Sun) to have come from any but a very young stellar body.

Brush's account of the disputes that Jean and Jeffreys had with Chamberlin remind one of more modern disputes. Chamberlin made a heroic attempt to bridge the gap between geology and astronomy by building a group around himself at Chicago. His dominant personality ensured success while he lived, but the group of talented individuals he had assembled dispersed after his death. In recent decades, the mantle has fallen to the California Institute of Technology, where the formula of combining nuclear physics, astrophysics and planetary sciences has been spectacularly successful.

Harold Urey was a leading figure in the 1950s through his insistence on the importance of explaining the chemical as well as the physical aspects of planetary formation. His influence inspired many workers, among them this reviewer, to work on planetary problems. By the 1970s the planetesimal hypothesis had become the modern intellectual framework for the origin of the Solar System, initially through the work of Victor Safronov in the former Soviet Union and then follow-

ing the adoption of the hypothesis by George Wetherill in the United States. Urey, Cameron, Ringwood and many others contributed to a resurgence of interest, partly driven by the Apollo landings on the Moon, which Urey had done so much to bring about.

The final section of the book deals with attempts to explain the origin of the Moon, and an entertaining story it is. The Apollo data demolished all previous theories in a spectacular demonstration of the strength of facts over ideas. The evolution of the giant impact theory for lunar origin is explained, with Brush correctly giving credit to Cameron who was the first to point out that the high angular momentum of the Earth-Moon system required a collision with the Earth of a body at least as massive as Mars. Previous collision hypotheses, employing smaller projectiles, missed this vital point.

At various places in these volumes, Brush discusses the roles of prediction and priority in discoveries. Among examples are early papers on giant lunar-forming impacts and on rings around Uranus that have been ignored. Readers will be interested in the reasons. Brush devotes space to many other topics: whether hypotheses should be testable, and the problems of studying the past, with asides on Karl Popper and on the evaluation of theories. The book is replete with references and personal accounts from more recent workers. Some of these later communications are more revealing than perhaps their authors intended. It is almost as dangerous to talk to historians as to reporters.

One stands in awe at the amount of scholarship recorded in these volumes. Only once did I detect a slip into the familiar historical trap of an unsupported statement: "Chamberlin... must have been indignant about [G. H. Darwin's] misrepresentation of his work but made no public protest". Textbook writers, who have a distressing tendency to reiterate familiar errors, would do well to study Brush's book, for it must now take its place as a primary source of information on the progress of science over the past 300 years.

In the tradition of good historical research, Brush sets the record straight on various issues. So we can now abandon the common, but erroneous, linking of the concept of the solar nebula to Kant and Laplace, and give credit to Laplace alone.

This major work of reference should be in all university and public libraries, as well as in departmental science libraries. It will remain an important document as long as planetary science is practised. □

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Mind traps

Stuart Sutherland

Kinds of Mind: Toward an Understanding of Consciousness. By Daniel C. Dennett. BasicBooks/Weidenfeld and Nicolson: 1996. Pp. 184. \$20, £11.99.

The Cerebral Code: Thinking a Thought in the Mosaics of the Mind. By William H. Calvin. MIT Press: 1996. Pp. 256. \$22.50, £14.95.

THERE should be a moratorium on books about consciousness. Although there is nothing new to say, philosophers and neuroscientists insist on saying it. In *Kinds of Mind*, Daniel Dennett presents an oblique approach to the topic. He writes almost entirely about minds, not consciousness, slipping in disparaging remarks here and there about his antagonists who have challenged his belief that consciousness is identical with certain aspects of the working of the brain.

The book is somewhat disconnected, although for the most part clear. Dennett begins by distinguishing the 'intentional stance' from the 'physical' and 'design' stances. In the physical stance, we determine what some entity will do by applying physical laws to its parts, a laborious process if we are deciding what move a chess-playing computer will make next. Alternatively, by knowing what an artefact, such as an alarm clock, is designed to do, we can — usually — decide what it *will* do in the future without making the elaborate calculations needed in the physical stance. In the intentional stance, we put ourselves in the position of the entity, regarding it as rational and purposive, and work out what we would do in the circumstances: hence we can predict, but with incomplete accuracy, the move the chess-playing computer will make next. Dennett avers that the application of mental terms to inanimate objects is legitimate: it should, however, be noticed that such anthropomorphism hindered the growth of science for many years.

Leaving aside the simplest organisms, he distinguishes three types of mind. Those that can learn to modify their behaviour in the light of the environment; those that mentally simulate possible courses of action and take that deemed optimal; and those that make use of tools — particularly language. The last group can vastly increase their intelligence by passing discoveries from one generation to the next. He rightly points out that the suffering caused by pain depends very much on the context and one's future prospects. A pain that one knows will disappear in five minutes is more bearable than one likely to last a lifetime or that presages death. So Dennett wonders whether it may be moral to cause pain to some animals and where the cut-off point is — house-flies, frogs or

cows? Needless to say, he can no more resolve this issue than anyone else.

Dennett ascribes consciousness to the highest type of mind — ours — and he seems unsure how far to attribute it to any other species, although he has previously announced that a highly intelligent computer would have it. But equating consciousness with complex information processing, whether carried out by the brain or by any other system, will not do. Dennett appears to be confusing consciousness with self-consciousness, but people are clearly conscious when they are not self-conscious, for example when they are engrossed in writing a book, when they are day-dreaming or when they are in terror because they are about to be shot.

Although the book has some amusing passages, such as that on the importance of the speed at which actions are performed for judgements of whether something is animate, it contains much that is doubtful and little that is new.

If there should be a moratorium on discussions of consciousness, there should be a complete ban on global speculations about the brain. The latest such exegesis, *The Cerebral Code*, is fearfully hard to understand. It is impossible to decide whether one should blame oneself for this failure because it contains something of substance or whether William Calvin's grandiose theory is in fact empty: after careful thought, I am inclined to adopt the latter view.

He starts with the function of the superficial pyramidal cells in the neocortex, claiming that when fired they will become entrained to one another through reciprocal recurrent connections. If any two are entrained, their synchronous firing will tend to entrain any other cell equidistant from both, forming a triangle of entrained cells. Because any two of these will fire yet another cell, an array of seven cells will give one another mutual support and will form a hexagon, made up of six adjoining equilateral triangles with bases out and a single cell at the central point. Through cells that descend and then ascend to a nearby part of the cortex, further hexagons could be formed at a distance from the original one. Calvin claims that object recognition could be achieved in this way with each of the areas firing in synchrony representing a different feature of the object (a rather antiquated view of shape recognition). With repeated firing, synapses are strengthened, and where this happens the hexagons (or triangles) become attractors (a buzzword). Different groups of hexagons compete both to control behaviour and for attention. How the read-out from the hexagons is achieved is unclear.

Calvin elaborates his ideas in great detail and claims to predict or explain almost everything worth predicting or explaining, from universal grammar to walking. Unfortunately, he omits to deal

with reinforcement, but doubtless with a little more tinkering the theory would cope with that. As befits a speculative theorist, Calvin's favourite expressions are "It may be that..." or "suppose that...", each introducing a further dubious presupposition. Until the theory has been simulated on a computer, it is impossible to know how, if at all, it would perform. In the meantime this book is likely to appeal only to the brave — or to the foolhardy. □

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Plants phantastica

Michael J. Balick

One River: Explorations and Discoveries in the Amazon Rain Forest. By Wade Davis. Simon and Schuster: 1996. Pp. 537. \$27.50.

RICHARD EVANS SCHULTES, a young graduate student interested in medicine, took a part-time job at Harvard Botanical Museum in 1934, working for the renowned economic botanist Oakes Ames. Schultes took Ames's course, "Plants and human affairs", a decision that changed his life, and botanical history, forever. The course introduced Schultes to the fascinating bond between

was devoted to stimulant and narcotic plants, and students were able to brew beer, distil alcohol, sample *Nicotiana rustica* (a potent relative of cultivated tobacco), imbibe caffeine from a variety of plant sources, taste kava-kava (a soporific plant used in the South Pacific) and examine many plants in the category "Phantastica" — plants "capable of causing excitation in the form of visions and hallucinations, often in colour". Schultes was required to prepare a report on a plant, and he chose the peyote cactus. So began his lifelong fascination with tropical botany and useful plants.

During the summer of 1936, at Ames's suggestion, Schultes travelled to Oklahoma with the ethnographer Weston La Barre to experience at first hand the ritual use of peyote — its tubercles are chewed by certain Indian tribes for their hallucinogenic effects. With Charlie Apekaum (Charlie Charcoal), a full-blooded Kiowa, as their guide, Schultes and La Barre visited 15 indigenous tribes and attended many peyote sessions. Schultes also studied with traditional healers such as Mary Buffalo, who shared her knowledge of Kiowa medicinal and religious plants. He returned to Harvard and wrote his senior thesis on peyote. Ames recognized in Schultes an extraordinary enthusiasm and intelligence, and encouraged him to pursue his curiosity about the role of plants in human affairs. The mentor's trust in the student did not go unrewarded.

One River is an extra-ordinary look into the lives and botanical explorations of Schultes and his protégé and most brilliant student, Timothy Plowman. The author, Wade Davis, has woven a biographical tapestry rich in history, adventure, intrigue and scholarship, during a six-year period of research that involved detailed interviews with Schultes and many of his colleagues, archival studies and first-hand field experience. It is doubtful that any student knows the details of his mentor's life and career as Davis does Schultes's, and this book is a most stimulating and inspiring look at the accomplishments of two great men.

One of Schultes's indigenous teachers in Sibundoy, Colombia, was a shaman named Salvador Chindoy, from whom Schultes learned of the use of many powerful plants. Many years later, when Plowman and Davis went to meet the now elderly Chindoy, Davis wrote: "There are moments in life that open wide the soul. Meeting Salvador Chindoy, as it turns out, was not one of them." Davis recounts their experiences with the elderly shaman, including their drinking binge with him, before they escaped from a "scene depressing beyond words". Not every shaman is a saint.

A fascinating chapter of Schultes's life is the time he spent surveying the rubber-producing potential of the northwest Amazon. The survey was part of the US



Schultes with members of the Makuna tribe from whom he learned of medicinal uses of rainforest plants.

people and plants, from prehistoric times to the present. Schultes went on to become the most distinguished ethnobotanist of our time.

A practical laboratory session, "directly engaging the students in some aspect of economic botany", followed each of Ames's lectures. The students made paper and ink, created perfumes from exotic oils, turned vegetable fats into soap, dyed clothing with leaves and roots, and studied botanical medicine. One of the sessions

effort during the Second World War to develop a source of this vital commodity to replace supplies from the plantations in South-East Asia, which were then under the control of the Japanese. The war effort depended on rubber — each Sherman tank contained half a ton of rubber, each bomber one ton and each battleship had 20,000 rubber parts weighing a total of 160,000 pounds (355,555 kg). Botanists were pressed into service in support of the war effort, for example in the collection of crucial raw materials such as quinine and rubber, species native to the neotropics.

The goals of the rubber programme were to estimate production that could be expected from wild rubber stands, to help support the initiation of production, and to collect seeds of important germplasm for a plantation industry to be established in the New World. Davis describes the botanical exploration, scientific productivity and extraordinary results that always characterized Schultes's work. As a result, germplasm collections, breeding programmes and rubber plantings were established in the Americas, ensuring that the United States and its allies would never again be held hostage to the supply of this vital commodity. Sadly, in 1954, Roy M. Hill, a State Department bureaucrat, succeeded in terminating the rubber programme, and the genetic material and plantations that Schultes and so many others had worked on were ploughed into the ground.

Today there is a new threat, biological rather than political, to the bulk of the world's rubber supplies — a leaf blight that could rapidly destroy the genetically homogeneous South-East Asian plantations. Unfortunately, the germplasm used to establish plantations in South-East Asia has no real resistance to the disease. As Davis states: "A single act of biological terrorism, the systematic introduction of fungal spores so small as to be readily concealed in a shoe, could wipe out the plantations, shutting down production of natural rubber for at least a decade. It is difficult to think of any other raw material that is as vital and valuable."

One River is an engaging and provocative read, a captivating story of past and present botanical exploration of the Amazon rainforest. Schultes and Plowman have served as mentors and role models for many tropical botanists, including this reviewer. This extraordinary book tells their story in a way that will inspire many others to carry on their work, at a time when the rate of loss of biological diversity and indigenous cultures has reached tragic proportions. □

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Flight from reason

Mark A. Norell and Luis M. Chiappe

The Origin and Evolution of Birds. By Alan Feduccia. Yale University Press: 1996. Pp. 420. \$65, £45.

ALAN Feduccia's new book is a revision of his widely read *Age of Birds*, first published in 1980. It is organized in a loose systematic framework, taking each of the main groups in turn with a few introductory and summary chapters on bird biology, origin and evolution. Coverage of fossil specimens is extensive, but the illustrations are largely recycled and the photographs murky. With recent fossil discoveries, renewed interest in avian palaeontology and advances in systematic methodology, it should be an important book.

Unfortunately, if one digs a little deeper the goblins emerge. Early on, Feduccia states that "cladistics does represent the most rigorous method for the analysis of morphology", and the book is rife with cladograms. Yet he applies a sort of self-critical evolutionary taxonomy for the 1990s that reflects a misunderstanding of modern phylogenetic methods. This ambiguous approach clouds and obfuscates nearly every discussion in the book. Take the following examples.

The author claims that "the semilunate carpal in *Archaeopteryx* has assumed great significance because a superficially similar element has been found in a handful of theropod dinosaurs and has thus been hailed by some paleontologists as a definitive character linking birds to a theropod ancestry". This wrist-bone is one of several characters supporting the hypothesis that birds are dinosaurs. Feduccia seems surprised that the presence of this bone in "only four types of dinosaurs" (actually many more) can be used as evidence for their relationship to birds. But he seems to miss the point that birds are not equally related to all dinosaurs but are preferentially related to a subset having this feature.

In his zeal to demonstrate that *Archaeopteryx* is "in the modern sense a bird", he fails to cite recent papers indicating that its gait was more similar to that of several non-avian dinosaurs than to birds, and that its wrist and other postcranial bones as well as the skull are substantially more primitive than their modern counterparts. And after giving an accurate account of the discoveries and life styles of the Enantiornithines, the most diverse group of Mesozoic birds, Feduccia says that "it is clear that *Archaeopteryx* is much more closely related to the Enantiornithes than it is to modern birds". Yet he fails to give references to the many cladistic analyses rejecting their purported close relationship.

The author relies too much on the fossil record. He states that "...nor is there any

substantial evidence that, with the exception of shorebirds, any modern avian groups extend past the Tertiary back into the Cretaceous" and that Mesozoic birds "underwent a dramatic and cataclysmic late Cretaceous demise". He does not warn readers (most of whom will not be palaeontologists) about just how spotty the Cretaceous fossil record is, and is careless in ignoring published work reporting modern avians in the Cretaceous. All this aside, on phylogenetic grounds, even the simple presence of shorebirds in the Cretaceous implies that many other modern taxa had already differentiated.

The same problem arises when the author states that the non-avian dinosaur relatives of birds are late Cretaceous in age, which, in his view, obviates the possibility that they could be related to the late Jurassic *Archaeopteryx*. This ignores recent discoveries of many members of these groups in coeval or nearly coeval beds. His logic also implies that humans and chimpanzees cannot be closely related because the earliest representatives of our own line appear more than 10 million years ago, whereas fossils on the chimp line are known only from the past million years or so.

Finally, Feduccia emphasizes similarities in function combined with 'special knowledge' ("intuitively pleasing" in his own words) as his basis for determining homology. Criticism of 'special knowledge' is self-evident. What is more, characters of vastly different function (such as tetrapod forelimbs or vertebrate postdentary bones) are homologues, and function has been deemed irrelevant to the issue of homology in modern systematics. Feduccia's misunderstanding of homology is clear in his discussion of the evolutionary importance of the furcula or wishbone. This element, homologous to the collarbones of other tetrapods, is present in birds and a variety of non-avian theropods. But Feduccia writes that "degeneration of the furcula with the evolution of flightlessness proves that it is intimately involved in the flight apparatus and argues strongly that whatever these structures are that are found in late Cretaceous dinosaurs, they are very likely not homologous with the furcula of birds". Obviously, to Feduccia the wishbones of non-avian theropods and birds cannot possibly be homologous simply because non-avian dinosaurs do not fly.

This book should have been much better; a well-illustrated volume detailing the history and biology of birds in a modern phylogenetic context is sorely needed. Until such a volume appears, we must still rely on the primary literature. □

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Constraints on mantle melting at mid-ocean ridges from global ^{238}U – ^{230}Th disequilibrium data

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The extent of radioactive disequilibrium between ^{238}U and its daughter product ^{230}Th in mid-ocean-ridge basalts from around the world is negatively correlated with axial ridge depth; local positive correlations with inferred mantle source composition are also observed. The larger ^{230}Th excesses seen in samples from shallow ridges reflect a larger melt contribution from a garnet-bearing source which may be explained by a deepening of the solidus in hotter mantle regions. These observations provide important constraints for modelling melt generation at mid-ocean ridges.

GLOBAL correlations between the chemistry of mid-ocean-ridge basalts (MORBs) and the axial depth of the ridge have been interpreted as reflecting variations in mantle temperature beneath ocean ridges^{1,2}. This model relies in part on the observation that major elements such as sodium and iron are not sensitive to minor source heterogeneities, except in the vicinity of hotspots³. This particular point has been the source of a controversy as some workers argue that variations in mantle composition have to be taken into account^{4,5} and propose an alternative model where MORB chemistry is controlled mostly by variable amounts of surface cooling⁴. Evaluation of radiogenic isotope and incompatible trace element data for MORBs also indicates the presence of long-lived heterogeneities in the MORB source mantle^{6–8}.

U-series disequilibrium offers a new perspective on this problem and is a particularly useful tool for investigating melting processes beneath the ridge. As the decay products of ^{238}U should be in secular equilibrium at the onset of melting (that is, $(^{230}\text{Th}/^{238}\text{U}) = 1$, where parentheses indicate an activity ratio), the initial $(^{230}\text{Th}/^{238}\text{U})$ of the source is known, in contrast to other geochemical tracers. As melting proceeds, the fractionation between ^{238}U and ^{230}Th will be controlled not only by partitioning of U and Th between melt and residual solid (as for all stable elements), but also by the rates of the melting process, as ^{230}Th is a short-lived isotope. Experimental determinations of U and Th partition coefficients in mantle minerals indicate that the two phases which control U–Th fractionation in the upper mantle are garnet and clinopyroxene^{9,10}. U is preferentially incorporated in garnet relative to Th, whereas in clinopyroxene, the reverse is true. Below the garnet–spinel transition (~ 20 kbar), a melt of typical mantle peridotite should be enriched in ^{230}Th relative to the parental ^{238}U (activity ratios greater than 1), whereas melts produced at shallower depths will not show this characteristic signature. U–Th disequilibrium thus provides a powerful probe for the depth of melting; here we use a global database to evaluate the controls on the production of mid-ocean-ridge basalts. The variations in $(^{230}\text{Th}/^{238}\text{U})$ for regionally averaged MORB data suggests a decreasing role in ^{230}Th – ^{238}U fractionation induced by garnet going from shallow to deep ridges. As garnet is a mantle phase that disappears above ~ 60 km, the larger ^{230}Th enrichment

relative to ^{238}U found at shallow ridges can be interpreted as a larger contribution from melts produced deeper in the mantle. We show here that $^{230}\text{Th}/^{238}\text{U}$ systematics in mid-ocean-ridge basalts are more likely to result from mantle temperature variations rather than variations in mantle source composition.

U–Th disequilibrium data for MORBs

The database we have used includes new mass-spectrometric U–Th disequilibrium analyses for samples from the extremes in axial depth in the global ridge system, from the shallow regions of the Mid-Atlantic Ridge (MAR) near the Azores¹¹ to the depths of the Australian–Antarctic discordance (AAD) and the MAR at 29–30° N (Table 1). These new data are crucial to the assessment of global MORB U–Th systematics in the context of melting processes beneath the ridge, and variations in source composition. Also included are new data from two ridge segments ('rift' and 'swell' regions¹²) near the Tamayo fracture zone on the East Pacific Rise (EPR; B.B., A.Z., C.H. and J. F. Bender, manuscript in preparation).

Here we have primarily considered samples that have been analysed by mass spectrometry, as results obtained by α -spectrometry have much larger analytical uncertainties and are more likely to be affected by manganese oxide contamination^{13–17} owing to the large amounts of sample that must be prepared (>3 g as compared to 50–300 mg of glass for mass spectrometry). The data we present (in all but Fig. 1b, where ridge-segment-averaged α -spectrometry data are shown for comparison), therefore, are associated with worst-case 2σ external uncertainties of 1–2%, and the precision and accuracy of the measurements account for very little of the observed variability.

The age control for MORB samples is also crucial. If the neovolcanic zone were a known single line on the sea floor, then a 500-m resolution in determination of the sample location would lead to an uncertainty of ~ 6 kyr for a fast-spreading ridge (half-spreading rate of 80 mm yr^{–1}), and ~ 50 kyr for a slow-spreading centre (half-spreading rate of 10 mm yr^{–1}). Thus, at relatively fast-spreading ridges, a stringent filter was applied to reject samples that are older than ~ 20 kyr. For some of these samples, there are also ^{226}Ra excess measurements that can be used to infer a maximum age of 8 kyr since eruption^{18–20}. At slow-spreading ridges, unless additional ^{226}Ra measurements are made, the age uncertainties are much larger than is desirable, but this does not affect the conclusions of this work.

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MORB U–Th systematics

$(^{230}\text{Th}/^{238}\text{U})$ activity ratios, which represent a measure of the recent fractionation between U and Th, range from 0.92 to 1.38 for the samples in our database. An activity ratio greater than 1 is most likely to be caused by melting in equilibrium with garnet. The low $(^{230}\text{Th}/^{238}\text{U})$ value of 0.92 for a 30°N Atlantic sample (replicated on two different aliquots), notably reflects a different sense of ^{238}U – ^{230}Th fractionation.

$(^{230}\text{Th}/^{238}\text{U})$ is plotted versus the axial depth of the ridge in Fig. 1. The axial ridge depth should reflect the thermal structure of the underlying mantle as it depends directly on the oceanic crustal thickness and the density and degree of melt depletion in the source mantle. A clear negative correlation is observed, suggesting that greater degrees of U–Th disequilibrium occur in MORBs erupted at shallower ridges. This is the opposite of what would be predicted in the context of simple batch melting, as shallower ridges with a thicker crust indicate greater extents of melting, which should cause less U–Th fractionation. In order to reduce the effect of local variability, as is observed for the Juan de Fuca ridge¹⁶, we have averaged the data for physically defined major ridge segments (Fig. 1b). In this figure, we have also included ridge-segment-averaged α -spectrometry data. Although the individual α -spectrometry data display a larger range of variability (see the 'error-bars' on Fig. 1b), the averaged data fall well within the trend defined by the mass-spectrometry data. We have also included data for Icelandic tholeiites in Fig. 1 (refs 21–25 and T.E., unpublished data) at an arbitrary axial depth of 0 m. Data for the very shallow Kolbeinsey ridge (0–1,000 m depth) span almost the entire range in $(^{230}\text{Th}/^{238}\text{U})$ for MORB, but the average would plot near the Icelandic tholeiites (C. Hémond, personal communication).

As both Th and U are very incompatible trace elements, the range in Th/U ratios observed in MORBs (2.0–3.6) is too large to be explained simply by differences in the degree of partial melting (5–20%) of a single source composition. Furthermore, as can be seen in Fig. 2a, many samples with the highest Th/U ratios have the largest ^{230}Th excesses ($(^{230}\text{Th}/^{238}\text{U})$ activity ratios greater than 1). There appears, therefore, to be some connection between the extent of U–Th disequilibrium and the Th/U of the source (Fig. 2a) which cannot be explained solely by melting-induced fractionation. This may be explained in the context of a model where recycled garnet pyroxenite or eclogite carries both the 'enriched' trace element signature and the U–Th fractionation potential^{26–30}. In contrast to the depth versus $(^{230}\text{Th}/^{238}\text{U})$ relationship, plotting ridge-segment averages does not improve the correlation; if anything, in fact, it tends to obscure it (data not shown). Thus, the trend shown in Fig. 2a may be more significant at the local scale than the global one. Figure 2b shows a plot of $(^{230}\text{Th}/^{238}\text{U})$ versus $\text{K}_2\text{O}/\text{TiO}_2$. The data are roughly consistent with the hyperbolic form of a mixing line between small-degree melts of a garnet-rich or eclogitic source, and higher-degree melts of a garnet-poor source, and thus further support the inferences drawn on the basis of the relationships observed in Fig. 2a.

Analysis of the present mass-spectrometric global database thus suggests potentially important roles for both thermal and compositional source variations in shaping the U–Th systematics of MORBs. There is a difficulty in readily resolving the two effects, because shallow ridges are frequently associated with isotopically enriched MORBs that are clearly related to compositional differences in their sources. We quantitatively examine, therefore, both 'thermal' and 'source' endmember hypotheses, before trying to assess their respective levels of importance.

Quantitative treatment of the thermal model

The axial depth– $(^{230}\text{Th}/^{238}\text{U})$ correlation can be interpreted qualitatively in the context of recent melting models for U–Th behaviour^{31–33}. At shallow ridges, the mantle is hotter and intersects its solidus at greater depths than would colder mantle. Lavas erupted at shallow ridges are, therefore, derived from a greater

mean depth of melting than those at deep ridges. Mineral partitioning data^{9,10} indicate that melting in the garnet stability field can most easily explain the ^{230}Th excesses observed in MORBs. We consider two endmember scenarios in our attempt to model the ^{230}Th -excess/axial-depth correlation: (1) a model where melt continues to equilibrate with the solid in the overlying melting column as it migrates toward the surface via porous flow³³; and (2) a dynamic melting model where melt is extracted into channels without any further interaction with the solid³². Both

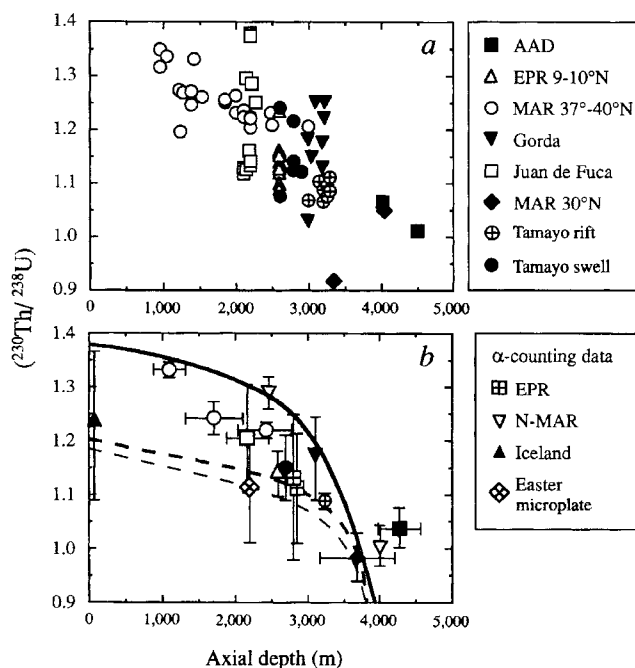


FIG. 1 a, $(^{230}\text{Th}/^{238}\text{U})$ versus axial depth. The largest ^{230}Th excesses are found at the shallowest ridges, while small degrees of disequilibrium are observed for deep ridges. Data sources are as follows: Juan de Fuca^{15,16}; Gorda¹⁶; EPR 9–10°N⁴⁹; MAR 37°N¹¹; MAR 30°N and AAD, this work; Tamayo, ref. 50 and B.B., A.Z., C.H.L. and J. F. Bender, manuscript in preparation. b, $(^{230}\text{Th}/^{238}\text{U})$ versus axial depth averaged over physically defined ridge segments. Within-sample variability is shown with 1σ error bars. Ridge-segment-averaged α -spectrometry data with uncertainties <10% (2σ) have also been included (data sources are as follows: Northern MAR (N-MAR)^{13,28}; EPR^{18,44}; Iceland^{21–25}; Easter microplate¹⁸). α -counting data with signs of contamination have also been discarded¹⁴. Note that these data do not significantly alter the trend defined by the mass-spectrometry data, although their variability, as shown by the error bars, is greater than for mass-spectrometry data. The quality of the correlation improves significantly with the averaging owing to elimination of local variability which may result from local source heterogeneities. The solid curve represents a model for $(^{230}\text{Th}/^{238}\text{U})$ and was calculated using the method in ref. 33. Along shallower ridges, diapirically ascending mantle intersects the solidus at higher pressure and, hence, greater U–Th fractionation takes place in the source owing to the enhanced presence of garnet. The matrix upwelling velocity is taken to be 2 cm yr⁻¹ and the matrix porosity, 0.1%. A melt productivity of 0.9% kbar⁻¹ is assumed. As the $^{230}\text{Th}/^{238}\text{U}$ in the melt is not very sensitive to the degree of melting in the upper part of the melting column, this figure can be tuned to match crustal production rates. Bulk partition coefficients for U and Th are, respectively, taken as 2.6×10^{-3} and 1.4×10^{-3} for garnet ilherzolite, and 1×10^{-4} and 1.5×10^{-4} for spinel ilherzolite^{9,10}. The garnet–spinel ilherzolite transition is fixed at 20 kbar. A deeper pressure for the spinel–garnet transition or a lower melt productivity than what is used here would push the curve to the left corner of the diagram. This may require some eclogite to be present in the source of MORBs. Based on the pressure of intersection of the solidus, the axial depth is then calculated with an isostatic model with a compensation depth of 200 km. The heavy dashed curve represents a model calculation with an upwelling velocity of 6 cm yr⁻¹ and the light dashed curve is obtained for a matrix porosity of 0.5% all else being equal.

TABLE 1 U–Th mass spectrometry data for representative MORB glasses

Sample number	Th (p.p.m.)	Axial depth (m)	(²³⁰ Th/ ²³² Th)*	±2σ	(²³⁸ U/ ²³² Th)	±2σ	(²³⁰ Th/ ²³⁸ U)
MAR 29–30° N							
OC180 D12-9	0.0955	3,350	1.2286	0.0087	1.3344	0.014	0.9207
OC180 D12-9 R	0.0941	3,350	1.2352	0.0105	1.3545	0.0153	0.9119
OC180 D14-1	0.1017	4,050	1.2402	0.0075	1.2011	0.0077	1.0325
OC180 D14-1 R	0.1059	4,050	1.2385	0.0088	1.1663	0.0061	1.0619
MAR 37° 30'–40° 30' N							
DR17-3	1.97	950	1.151	0.0067	0.8536	0.0014	1.348
RC89	1.89	1,538	1.117	0.014	0.8858	0.0040	1.261
DR26-1	0.467	2,100	1.107	0.0046	0.8968	0.0037	1.234
DR24-2	0.677	3,000	1.034	0.0049	0.8574	0.0035	1.205
AAD							
D22-13	0.146	4,000	0.931	0.015	0.87481	0.008	1.065
D29-5	0.222	4,300	1.104	0.025	1.09187	0.015	1.011
EPR 22–23° N Tamayo							
D7-36 (swell)	0.033	2,700	1.676	0.013	1.378	0.009	1.216
975-5-1 (rift)	0.104	3,200	1.498	0.010	1.393	0.018	1.075

* Ratios in parentheses are activity ratios.

models are sensitive to mantle upwelling velocities, as this parameter controls the amount of ²³⁰Th produced by decay during the melting process. The work of Iwamori³⁴ and Richardson and McKenzie³⁵, however, suggests that in the case of passive spreading the mantle upwelling velocity is independent of spreading rate, and rather uniform for the global ridge system. Although this may not be completely true in the case of more active upwelling, the predicted increase in upwelling velocity does not exceed a factor of 2 for half-spreading rates ranging from 1 to 6 cm yr⁻¹ (ref. 36). We have therefore used an upwelling velocity of 2 cm yr⁻¹ to represent the whole ridge system. Larger upwelling velocities may occur beneath ridge-centred hotspots, such as Iceland, and some of the noise in the data relative to the model curve may indeed relate to this parameter.

We have first modelled the ²³⁰Th/²³⁸U versus axial depth correlation using the melting model of ref. 33 with re-equilibration during ascent, setting the range in solidus pressure for the upwelling mantle (re-equilibration is in fact not a requirement for the section of the melting column where garnet is not present). Our calculations suggest a total variation in solidus pressure from ~35 kbar for shallow ridges to less than 15–20 kbar for the deepest ridges (see model curve in Fig. 1a; values used for relevant model parameters are given in Fig. 1 legend). The ²³⁸U excess found in sample OCE180 D12-9 from the MAR can be explained as resulting from melting which starts in the spinel lherzolite field ($P_0 < 20$ kbar, where P_0 is the solidus pressure). The lower limit of solidus pressure cannot be precisely constrained by the calculation as (²³⁰Th/²³⁸U) is rather insensitive to P_0 for $P_0 < 20$ kbar. This difference in the initial depth of melting could imply a variation in mantle temperature of at least 200 °C. In principle, however, these results need not bear directly on mantle temperature, as other factors (such as water content) that could cause large variations in the initial pressure and pressure interval of melting might also account for the observations.

In contrast to the Spiegelman and Elliott³² genre of models, simple dynamic melting models do not predict an increase in excess ²³⁰Th as the pressure of intersection of the solidus deepens, or as melting extends to a greater depth below the garnet-spinel transition^{29–32}. This stems from the fact that because U is rapidly stripped out of the residual solid in dynamic melting models, 'in-growth' of ²³⁰Th occurs only in a tiny portion of the melting column just after the onset of melting. Thus, the presence or absence of garnet above this small zone is of no consequence to U–Th disequilibrium. However, one might speculate that the bulk distribution coefficients increase with pressure and, in this case, dynamic melting at deeper mantle levels would yield higher ²³⁰Th

excesses. A range of (²³⁰Th/²³⁸U) from 1.10 to 1.30 can be obtained with a variation in bulk crystal–liquid partition coefficient of a factor of ~4 for both U and Th over a pressure range of 19 to 29 kbar.

For the high degrees of melting in MORB, ²³⁰Th excess, as modelled above, is not produced by net fractionation of Th from U in the erupted melt relative to the source but from in-growth of ²³⁰Th during the melting process. Such behaviour is only applicable to short-lived nuclides, and so ²³⁰Th excesses are not necessarily expected to correlate with indices of garnet fractionation inferred from stable geochemical tracers. The effects of extending the model to two dimensions does not substantially alter the position of the model curves. Slightly larger ²³⁰Th excesses can result if small-degree melts produced far from the ridge axis are brought instantaneously to the surface.

²³⁰Th/²³⁸U and source enrichment

There are two distinct types of source enrichment that could have an effect on U–Th disequilibrium. The first is the water content of the source. Water lowers the solidus, and hence leads to a greater depth of melting, and a greater garnet influence. Increasing water content is difficult to distinguish from mantle temperature, because both increase the solidus pressure, and it appears that water may not have a major independent effect on the major-element compositions of mantle melts³⁷. In any case, both mantle temperature and water have the commonality of increasing depths and extents of melting associated with greater ²³⁰Th excess. But if a higher water content is associated with shallower ridges, the melting environment may become suitably more oxidizing so that U would be more incompatible than Th, leading to smaller ²³⁰Th excesses for those ridges.

The second way that source chemical composition could be related to the degree of U–Th disequilibrium produced in derivative melts would be through an association of chemical composition with modal mineralogy. Such an association could be produced by the recycling of oceanic crust into the MORB source, which would lead to the presence of geochemically enriched, eclogitic material in the mantle^{38–41}. As the mode of garnet is higher in eclogite than in lherzolite, a higher relative proportion of the eclogite could lead to a larger ²³⁰Th excess in the melt³⁰. The correlation between (²³⁰Th/²³⁸U) and Th/U could then be explained by varying contributions from enriched eclogitic material with high Th/U and K₂O/TiO₂ to the total melt (Fig. 3a). Even in this context, batch melting cannot explain the observed disequilibrium as the bulk partition coefficients for U and Th in the eclogite-peridotite mixture would still not be large

enough to fractionate ^{230}Th from ^{238}U for degrees of melting greater than $\sim 5\%$. However, rather large degrees of disequilibrium can be produced during transport if the melting rate is slow enough to allow significant in-growth of ^{230}Th while ^{238}U is retained in the garnet-bearing residue^{9,10} (Fig. 3a). This possibility has been modelled using two limiting cases of the equilibrium-percolation model of Spiegelman and Elliott³³ (Fig. 3b). In the first case, the extraction time is considered to be long relative to the half-life of ^{230}Th ; in the second case, the extraction is fast. Also shown in Fig. 3b is the result for a dynamic melting model with instantaneous extraction from an eclogite source. The model calculations show that it is possible to produce ^{230}Th excesses as large as 50% by mixing melts of eclogite and lherzolite in the case of rapid extraction, assuming no re-equilibration between the eclogite melt and the solid lherzolite before mixing. Variable amounts of eclogitic melt can be mixed into the total melt, imparting a high Th/U and high $(^{230}\text{Th}/^{238}\text{U})$ to the pooled melt (Fig. 3b). Hence, both mineralogical and volatile source heterogeneities that are likely to occur in the mantle may have an influence on U–Th disequilibrium systematics.

Origin of the garnet signature in MORBs

The problem we face, therefore, is to try to separate the effects of mantle thermal structure and source composition on the degree of U–Th disequilibrium produced in MORBs. Evaluation of the problem is complicated by the fact that some degree of coupling between source composition and thermal structure is very likely, as the shallowest regions of the mid-ocean-ridge system are often found to be in the proximity of hotspots. In this context, a clear association of source composition with solidus pressure, by virtue

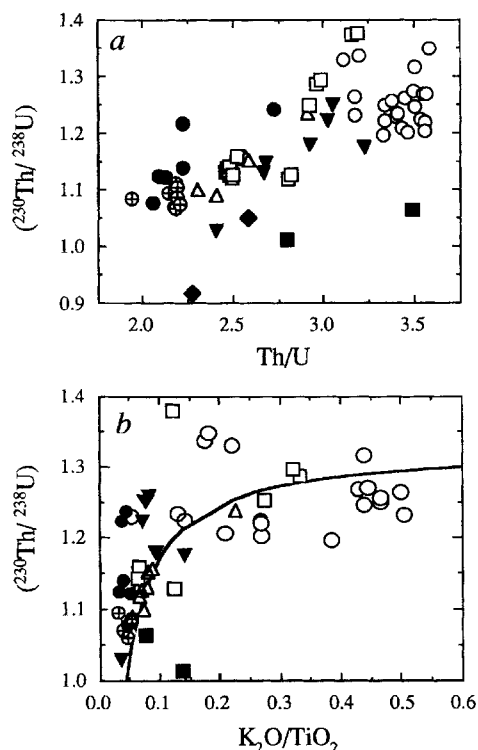


FIG. 2 a, $(^{230}\text{Th}/^{238}\text{U})$ versus Th/U measured in MORB glasses. Symbols as in Fig. 1. The broad positive trend indicates that ^{230}Th excess is more pronounced in melts of sources with higher Th/U ratios. Note also the well defined local trends for some of the sample suites. b, $(^{230}\text{Th}/^{238}\text{U})$ versus $\text{K}_2\text{O}/\text{TiO}_2$ for MORB glasses. Symbols as in Fig. 1. Overall, the data form a hyperbolic trend which could result from mixing of melts formed from garnet-rich and garnet-poor sources. Individual localities do not always display coherent trends.

of variable amounts of recycled eclogite or garnet pyroxenite in the source, should lead to simple correlations between parameters indicative of source enrichment and axial depth. There is no correlation in our data set between Th/U or $\text{K}_2\text{O}/\text{TiO}_2$ and ridge depth. But, although there are no simple global-scale systematics between isotopic and trace-element indicators of enrichment and ridge depth, there are well known correlations around hotspots between depth and geochemical enrichment (see, for example, ref. 42), and more complex correlations exist for larger regions⁴³. It is clear that, on this scale, the effects of mantle temperature and mantle heterogeneity can be very difficult to separate.

Nevertheless, the compositional model fails, in detail, to explain important features of the global U–Th data set. For example, individual, well characterized localities, where enrichment signatures can be accurately predicted, also fail to display simple correlations between enrichment parameters and $(^{230}\text{Th}/^{238}\text{U})$. The MAR near the Azores, for example, displays no correlation between $^{206}\text{Pb}/^{204}\text{Pb}$ and $(^{230}\text{Th}/^{238}\text{U})$, although we know that $^{206}\text{Pb}/^{204}\text{Pb}$ should be a good indicator of mixing

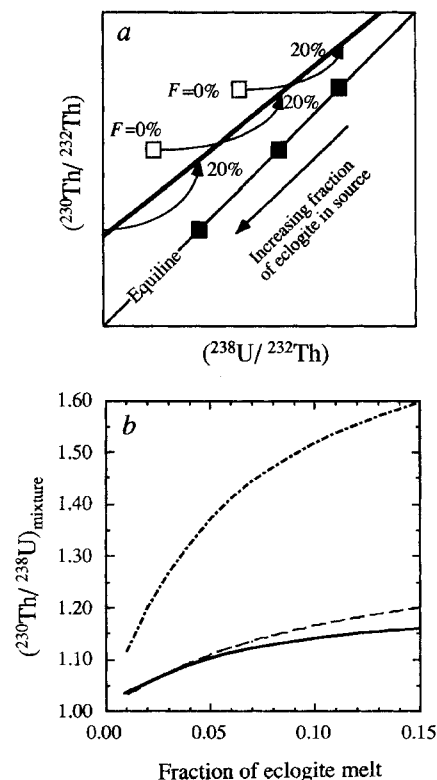


FIG. 3 a, Cartoon showing the potential effect of source composition on $(^{230}\text{Th}/^{238}\text{U})$. Melts of sources with a relatively higher proportion of a garnet-rich, chemically enriched component (that is, high Th/U) develop correspondingly larger ^{230}Th excesses. The filled squares represent the mantle source composition in secular equilibrium; the heavy solid line represents the global trend for MORB; curved arrows denote pooled melts with F , the degree of melting, ranging from 0 to 20% (the open squares represent 0%, and the arrowheads represent 20%). A transport melting model is used. b, Model calculation of $(^{230}\text{Th}/^{238}\text{U})$ in a mixture of peridotite melt and eclogite melt. The peridotitic melt is assumed to be in secular equilibrium. The dot-dashed curve denotes the maximum ^{230}Th excess obtained if the melt extraction is fast, whereas the solid curve represents the case of slow melt extraction (equations from Spiegelman and Elliott³³). Various mixtures of the two melts can produce the range of $(^{230}\text{Th}/^{238}\text{U})$ found in MORB. The long-dashed curve shows the result of a dynamic melting calculation using the model of ref. 32. The input parameters are the same as given in Fig. 1 legend.

between the Azores plume and normal sub-ridge mantle (ref. 11 and L. Dosso, personal communication). Conversely, Sr and Pb isotope ratios are nearly constant at the Juan de Fuca and Gorda ridges, but these ridges are characterized by a large degree of variation in ($^{230}\text{Th}/^{238}\text{U}$) and Th/U ratios¹⁶ (what little range there is in $^{87}\text{Sr}/^{86}\text{Sr}$ does not correlate with excess ^{230}Th). These variations would have to be related to melting of a heterogeneous mantle source⁴¹ whose signature is not visible in the radiogenic isotopes⁴⁴. Local effects such as these may be filtered by ridge-segment-scale averaging, as we have done for the data in Fig. 1b.

The compositional model is also not supported by systematics of volcanics sampled at small seamounts adjacent to the EPR⁴⁵. These samples offer the opportunity to test the effect of source heterogeneity in the absence of any thermal variability. The seamount samples are characterized by enriched isotopic signatures and were argued to represent melts of an eclogite- or amphibole peridotite-bearing source^{8,46}. The samples we analysed have been dated by (U + Th)/He (ref. 47) and all have extremely low initial ^{230}Th excesses or ^{238}U excesses⁴⁵. If the U/He ages are reliable, the data suggests that the putative enriched source of the EPR seamounts does not produce large ^{230}Th excesses. This seamount data may or may not have any direct bearing on the influence of eclogite on U–Th systematics beneath the ridge, but it certainly does not argue for the compositional model.

Although the thermal model is more appealing—owing to the quality of the correlation of ($^{230}\text{Th}/^{238}\text{U}$) with axial depth, and the fact that averaging tends to improve it—it too suggests some complexities. Kolbeinsey, a shallow ridge with a relatively depleted geochemical signature, could be considered as an important test for this model, and the indications are that it would fall slightly below the global trend (C. Hémond, personal communication). This could be ascribed to the very rapid mantle upwelling beneath Iceland (clearly, a very different situation than the

MAR near the Azores), which might lead to smaller ^{230}Th excesses. As Kolbeinsey is considered to be more depleted, in general, than Iceland and displays a similar degree of ^{230}Th – ^{238}U disequilibrium, this favours the idea that the lower ^{230}Th excesses at Iceland are related to faster upwelling rather than a source effect. Such could also be the case for the Easter microplate samples¹⁸ which seem to be offset from the global trend. Despite an inferred shallow depth of melting for the AAD lavas¹, these samples show a weak garnet signature that could be attributed to the presence of some eclogite in the source at that locality.

The most robust aspect of the models discussed here is that the degree of U–Th disequilibrium must be related to the extent to which melting takes place in the presence of garnet. The systematics of the data suggest that, on a global scale, the enhanced presence of garnet is largely explained by a deepening of the solidus in hotter mantle regions which are associated with shallower ridges. It is difficult at this stage to comment definitely on the effects of active versus passive upwelling. This suggests the need for detailed studies of restricted geographical regions and the use of other tracers such as ^{231}Pa and ^{226}Ra , which are more sensitive to upwelling rates²⁹.

The U–Th disequilibrium data is, then, on a global scale, consistent with the model of Klein and Langmuir¹ in which major-element systematics are controlled by the initial depth of melting beneath the ridge. Were the final depth of melting variable, due to surface cooling, and the initial depth of melting constant⁴, a positive correlation between ($^{230}\text{Th}/^{238}\text{U}$) and ridge depth would be expected, in stark contrast to the negative correlation that is observed. Rare-earth element and Lu/Hf data⁴⁸ suggest a more important role for garnet fractionation at deep ridges than at shallow ridges, a notion which contradicts models based on major elements and U–Th disequilibrium. A self-consistent melting model for mid-ocean ridges will have to integrate all of these observations. □

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- Klein, E. M. & Langmuir, C. H. *J. Geophys. Res.* **92**, 8089–8115 (1987).
- Langmuir, C. H., Klein, E. M. & Plank, T. in *Mantle Flow and Melt Generation at Mid-ocean Ridges* (eds Phipps Morgan, J., Blackman, D. K. & Sinton, J. M.) 183–280 (Am. Geophys. Union, Washington, 1992).
- Langmuir, C. H. & Hanson, G. N. *Phil. Trans. R. Soc. Lond. A* **297**, 383–407 (1980).
- Shen, Y. & Forsyth, D. W. *J. Geophys. Res.* **100**, 2211–2238 (1995).
- Albarède, F. *J. Geophys. Res.* **97**, 10997–11009 (1992).
- Hart, S. R., Schilling, J.-G. & Powell, J. L. *Nature* **246**, 104–107 (1973).
- White, W. M. & Schilling, J.-G. *Geochim. Cosmochim. Acta* **42**, 1501–1516 (1978).
- Zindler, A., Staudigel, H. & Batiza, R. *Earth Planet. Sci. Lett.* **70**, 175–195 (1984).
- Beattie, P. D. *Nature* **363**, 63–65 (1993).
- LaTourrette, T. Z., Kennedy, A. K. & Wasserburg, G. J. *Science* **261**, 739–742 (1993).
- Bourdon, B., Langmuir, C. H. & Zindler, A. *Earth Planet. Sci. Lett.* **142**, 175–189 (1996).
- Bender, J. F., Langmuir, C. H. & Hanson, G. N. *J. Petrol.* **25**, 213–254 (1984).
- Condomines, M., Morand, P. & Allègre, C. J. *Earth Planet. Sci. Lett.* **55**, 247–256 (1981).
- Reinitz, I. & Turekian, K. K. *Earth Planet. Sci. Lett.* **94**, 199–207 (1989).
- Goldstein, S. J., Murrell, M. T. & Janecky, D. R. *Earth Planet. Sci. Lett.* **96**, 134–146 (1989).
- Goldstein, S. J., Murrell, M. T., Janecky, D. R., Delaney, J. R. & Clague, D. A. *Earth Planet. Sci. Lett.* **107**, 25–41 (1991).
- Zindler, A. et al. *Anal. Chem.* (submitted).
- Rubin, K. & MacDougall, J. D. *Nature* **335**, 158–161 (1988).
- Rubin, K. & MacDougall, J. D. *Earth Planet. Sci. Lett.* **101**, 313–322 (1990).
- Volpe, A. & Goldstein, S. J. *Geochim. Cosmochim. Acta* **57**, 1233–1242 (1993).
- Condomines, M., Morand, P. & Allègre, C. J. *Earth Planet. Sci. Lett.* **55**, 393–406 (1981).
- Hémond, C. et al. *Earth Planet. Sci. Lett.* **87**, 273–285 (1987).
- Nicholson, H., Condomines, M. & Fitton, J. G. *J. Petrol.* **32**, 1005–1050 (1991).
- Sigmarsson, O., Condomines, M. & Gronvold, K. *Geophys. Res. Lett.* **18**, 2229–2232 (1991).
- Sigmarsson, O., Condomines, M. & Fourcade, S. *Contrib. Mineral. Petrol.* **112**, 20–35 (1992).
- Chabaux, F. & Allègre, C. J. *Earth Planet. Sci. Lett.* **126**, 61–74 (1994).
- Hirschmann, M. & Stolper, E. M. *Geol. Soc. Am. Abstr. Prog.* **26(7)**, 38 (1994).
- Ben Othman, D. & Allègre, C. J. *Earth Planet. Sci. Lett.* **98**, 129–137 (1990).
- Lundstrom, C. C., Gill, J., Williams, Q. & Perfit, M. R. *Science* **270**, 1958–1961 (1995).

- Hirschmann, M. & Stolper, E. M. *Contrib. Mineral. Petrol.* **124**, 185–208 (1996).
- McKenzie, D. *Earth Planet. Sci. Lett.* **72**, 149–157 (1985).
- Williams, R. W. & Gill, J. B. *Geochim. Cosmochim. Acta* **53**, 1607–1619 (1989).
- Spiegelman, M. & Elliott, T. *Earth Planet. Sci. Lett.* **118**, 1–20 (1993).
- Iwamori, H. *Earth Planet. Sci. Lett.* **125**, 1–16 (1994).
- Richardson, C. & McKenzie, D. *Earth Planet. Sci. Lett.* **128**, 425–437 (1994).
- Sparks, D. W., Parmentier, E. M. & Phipps Morgan, J. J. *Geophys. Res.* **92**, 8089–8115 (1987).
- Hirose, K. & Kawamoto, T. *Earth Planet. Sci. Lett.* **133**, 463–475 (1995).
- Hanson, G. N. *J. Geol. Soc. Lond.* **134**, 235–253 (1977).
- White, W. M. & Hofmann, A. *Nature* **296**, 821–825 (1982).
- Allègre, C. J. & Turcotte, D. L. *Nature* **323**, 123–127 (1985).
- Prinzhofer, A., Lewin, E. & Allègre, C. J. *Earth Planet. Sci. Lett.* **92**, 189–206 (1989).
- Schilling, J.-G. *Nature* **242**, 565–571 (1973).
- Zindler, A. & Salters, V. in *Plume 2* (eds Anderson, D. L., Hart, S. R. & Hofmann, A. W.) 166–169 (Terra Nostra Vol. 3, Alfred-Wegener-Stiftung, Bonn, 1995).
- Rubin, K. & MacDougall, J. D. *Earth Planet. Sci. Lett.* **114**, 149–157 (1992).
- Zindler, A. et al. *Trans. Am. Geophys. Un.* **73**, 375 (1992).
- Graham, D. W. et al. *Contrib. Mineral. Petrol.* **99**, 446–463 (1988).
- Graham, D. W., Jenkins, W. J., Kurz, M. D. & Batiza, M. D. *Nature* **326**, 384–386 (1987).
- Salters, V. J. M. *Earth Planet. Sci. Lett.* **141**, 109–123 (1996).
- Goldstein, S. J., Murrell, M. T. & Williams, R. W. *Earth Planet. Sci. Lett.* **115**, 151–159 (1993).
- Bourdon, B. thesis, Columbia Univ. (1994).

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Development, plasticity and evolution of butterfly eyespot patterns

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The developmental and genetic bases for the formation, plasticity and diversity of eyespot patterns in butterflies are examined. Eyespot pattern mutants, regulatory gene expression, and transplants of the eyespot developmental organizer demonstrate that eyespot position, number, size and colour are determined progressively in a developmental pathway largely uncoupled from those regulating other wing-pattern elements and body structures. Species comparisons and selection experiments suggest that the evolution of eyespot patterns can occur rapidly through modulation of different stages of this pathway, and requires only single, or very few, changes in regulatory genes.

ANIMAL colour patterns are often adaptations to different environments and predators, and their diversity represents one important form of morphological evolution^{1,2}. A key challenge in evolutionary biology is to understand how phenotypic diversity is generated by the modification of developmental pathways. Butterfly wings are decorated with a great variety of scale colour patterns, which are subject to strong natural selection and are amenable to genetic and developmental study³. Extensive analyses of the monophyletic Lepidoptera indicate that roughly 15,000 species wing patterns have evolved within ~100 Myr by modifications to a common set of pattern elements. Dramatic diversity in wing pattern also occurs within some butterfly species, such as the African satyrine *Bicyclus anynana*, which shows phenotypic plasticity whereby individuals of the same genotype develop into alternative phenotypes through hormone-mediated responses to the environment³⁻¹⁰. Understanding the regulation and evolution of plasticity may offer general insights into the genetic and developmental bases of morphological evolution¹¹⁻¹⁴.

One important pattern element on butterfly wings, the eyespot, arises through the establishment of an inductive organizing centre (the focus) at a specific location on the developing wing¹⁵. Transplantation of the focus can induce surrounding host cells to form an eyespot in an ectopic site^{15,16}. The focus is proposed to be a signalling source for a morphogen, the levels of which determine the pigmentation of surrounding cells^{3,15}. Recent investigations have indicated that regulatory genes, such as the *Distal-*

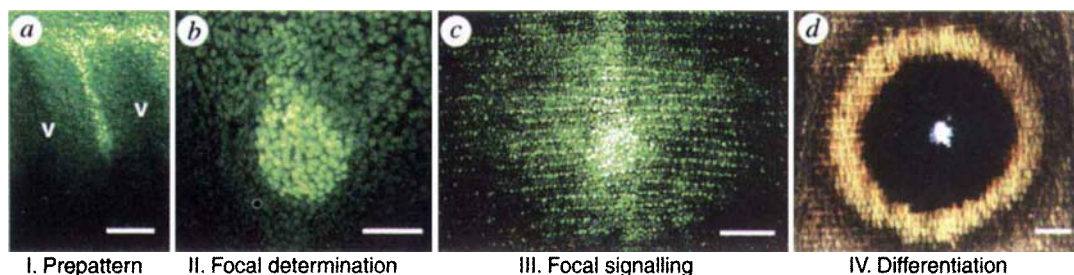
less homeobox gene, are specifically expressed in the eyespot focus¹⁷.

One advantage for studying the eyespot focus is that the colour patterns are far less constrained than those regulated by other organizers acting earlier in development. Eyespots vary tremendously in number and pattern, reflecting the subtle, varying and strong visual selective pressure under which butterflies live. To understand how these patterns are generated and diverge, we first define the developmental pathway that regulates eyespot formation. We then examine how this developmental pathway is modulated in species bearing different numbers of eyespots, or no eyespots at all. Finally, we analyse in detail the genetic and developmental bases for the phenotypic plasticity of eyespot size in alternative seasonal forms and artificially selected lines of *B. anynana*.

The eyespot developmental pathway

We have performed molecular, genetic and transplantation studies which suggest that eyespot position, number, size and colour are determined progressively in four stages. The first three stages of eyespot formation involve the placement of, and signalling from, the organizing focus, and are accurately reflected by the expression of the *Distal-less* (*Dll*) regulatory gene (Fig. 1a-c). An antibody to the *Dll* protein was used to follow the dynamics of *Dll* expression in *B. anynana* during the late larval and early pupal stages, when eyespots are determined. In the imaginal discs of

FIG. 1 Expression of *Distal-less* protein marks the eyespot foci and reveals three stages of eyespot formation. a-c, Ontogeny of the *Dll* protein pattern within a single wing subdivision and eyespot field of *B. anynana*. a, *Dll* expression in wing disc of mid-fifth instar larvae is highest in the middle of each wing subdivision (V, veins). b, By the late fifth instar, the protein accumulates at high levels in the future centre of the eyespot. c, By 24 h after pupation, when scale-forming cells (large nuclei) are aligned into rows, *Dll* is expressed in the larger scale-forming cells and



the epithelial cells in the centre of the field which will form a large portion of the future eyespot field (d). Scale bars: a, b, 100 μm; c, 50 μm; d, 0.5 mm.

early fifth-instar larvae, *Dll* is expressed in a broad distal band and at high levels in stripes down the midline of each subdivision (stage I, the larval prepattern; Fig. 1a). At the proximal tips of these stripes, the *Dll* pattern begins to enlarge. In each subdivision of the hindwing imaginal disc (but in only two subdivisions of the forewing), the stripes then resolve into stable circular spots of *Dll* expression by the late fifth-instar stage (stage II, focal determination; Figs 1b, 2a). In the early pupa, strong *Dll* expression expands to encompass a broader circular field of the scale-forming cells, which are now aligned in rows and protrude above the other epithelial cells (Fig. 1c). We believe this expanded domain reflects a response to signalling from the epithelial focus, which remains visible at the centre of the field of *Dll*-expressing cells (stage III, focal signalling; Fig. 1c). We deduce that the *Dll* spots must include the inductive focus, because grafts containing them induce ectopic eyespots (see Fig. 3 below). The positions of these spots of *Dll* expression correspond to the central regions of the eyespots that form several days later when scale colour differentiation occurs (stage IV, differentiation; Fig. 1d). Because *Dll* expression reveals the larval prepattern, accurately marks the position and number of foci, and reflects signalling from the focus, we have used it to analyse eyespot formation in mutant butterflies and different species, morphs and selected lines.

The genetics of eyespot patterns

To better define the stages at which eyespot formation is regulated, and to identify some of the genetic determinants involved, we have begun to isolate and analyse mutants with altered eyespot patterns. Three spontaneous autosomal dominant *B. anynana* mutants (*Cyclops*, *Spotty* and *Bigeye*) selectively affect eyespot position, number and size, and perturb three different stages of eyespot formation.

Heterozygotes for *Cyclops* are dramatically altered in the position and number of eyespots on the hindwing (homozygotes die as embryos). Wild-type hindwings normally bear seven distinct eyespots (Fig. 2b), but *Cyclops* heterozygotes have abnormal wing venation and display a range of phenotypes from a loss and/or fusion of one or two eyespots (data not shown) to the formation of one large elliptical eyespot and the loss of the other eyespots (Fig. 2f). However, even in severely disrupted hindwings, pattern elements that are proximal or distal to the eyespots are largely

unaffected (Fig. 2f).

To determine when and how development is changed in the *Cyclops* mutant, we examined *Dll* expression in mutant imaginal discs. The broad distal band of *Dll* expression seems normal, but the larval prepattern (stage I) and the foci (stage II) are severely disrupted. Specifically, regular larval midline stripes of *Dll* expression are not observed (data not shown), and individual focal spots are absent or greatly elongated to four or five times their normal cell number (Fig. 2e). The distortion of the *Dll* pattern and adult eyespot shape and venation suggests that the *Cyclops* mutation might involve a gene required for some aspect of patterning along the anteroposterior axis.

Heterozygotes and homozygotes for the *Spotty* mutation¹⁸ display two additional eyespots on the adult forewing (compare Fig. 2d and 2h), but other elements of the forewing and hindwing pattern appear normal. In forewing imaginal discs of *Spotty* homozygotes, no disruption of the larval prepattern was evident (stage I), but extra *Dll*-expressing focal spots were detected in the larval fifth-instar disc (stage II; data not shown) and in the pupa (Fig. 2g), demonstrating that the *Spotty* mutation causes the establishment of specific extra foci during forewing development.

Homozygotes for the *Bigeye* mutation display a dramatic enlargement of eyespot size, with the most pronounced effects on the ventral hindwing (Fig. 2j) and ventral forewing. Analysis of *Dll* expression reveals no change in the number or size of the *Dll*-expressing foci in larval (Fig. 2i) or early pupal wings (data not shown). Thus the effect of *Bigeye* on eyespot size is likely to be downstream of the establishment of and signalling from the focus (after stage III) and may involve the response to focal signalling.

Positional information and eyespot colour

The above results demonstrate that discrete genes influence eyespot position, number and size, but other determinants must influence the ultimate colour pattern of the eyespots induced by different foci both within and between species. In experiments with a different butterfly species, *Precis coenia*, in which we surgically transplanted small pieces of early pupal wing tissue containing the *Dll*-expressing cells to ectopic sites, the colour pattern of surrounding host cells was found to depend on their proximodistal position.

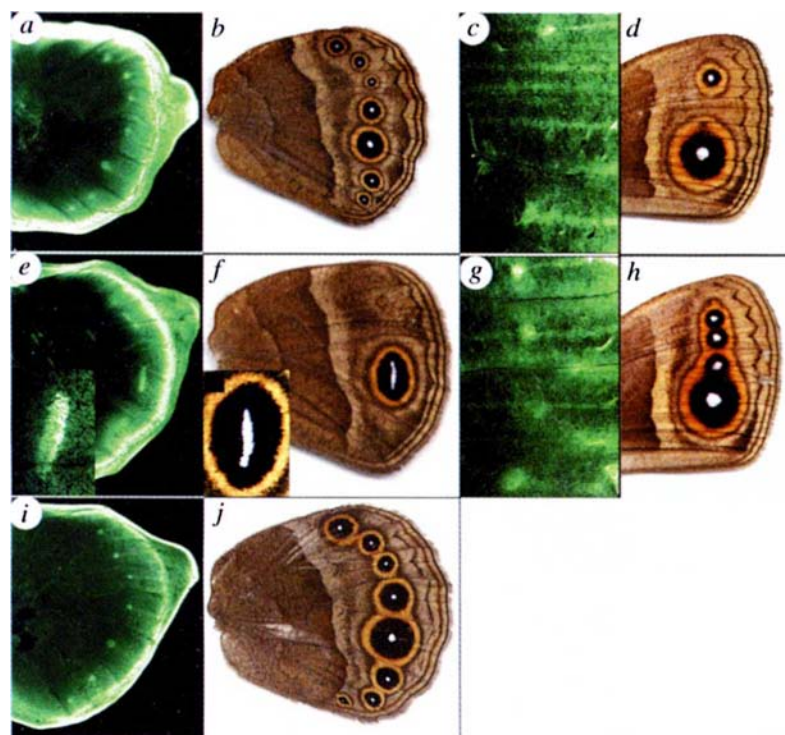


FIG. 2 The *Cyclops*, *Spotty* and *Bigeye* mutants affect eyespot position, number and size at three different stages in the eyespot developmental pathway. a, *Dll* expression in a late-fifth instar *B. anynana* ventral hindwing imaginal disc is in a broad distal band and at high levels in seven spots which correspond to the future position of the seven eyespots (b) that form on the adult ventral hindwing. c, Wild-type *B. anynana* forewing (ventral view) 24 h after pupation. Two *Dll* spots are visible as scale cells form (arrows); these correspond to the future position of the two eyespots that form on the adult forewing (d). e, *Dll* expression in a *Cyclops* mutant hindwing imaginal disc is in all distal cells, as in the wild type, but short stripes of high-level *Dll* expression arise in place of the circular spots (inset). f, The strong *Cyclops* mutant phenotype involves the formation a large elliptical eyespot with an elongated white focus (inset) and the loss of other eyespots (compare with b)). The marginal and sub-marginal bands and the proximal band (which are elements of different patterning systems to the eyespots; see ref. 3) are largely unaffected. g, In *Spotty* mutants, two additional *Dll* spots form between the wild-type spots, in the future position of the additional eyespots that form on the adult forewing (h). i, *Dll* expression in the *Bigeye* mutant wing disc is indistinguishable from the wild-type; j, much larger eyespots form on the ventral hindwing of *Bigeye* adults (compare to b).

When *P. coenia* focal grafts were transplanted to a nearby distal position (see Fig. 3a for location of grafts), an ectopic eyespot was induced that had buff-coloured scales surrounding the graft (Fig. 3b; see also ref. 15). By contrast, when a focal graft was transplanted to a more proximal site in the same wing subdivision, a ring of scales coloured bright orange was induced around the graft

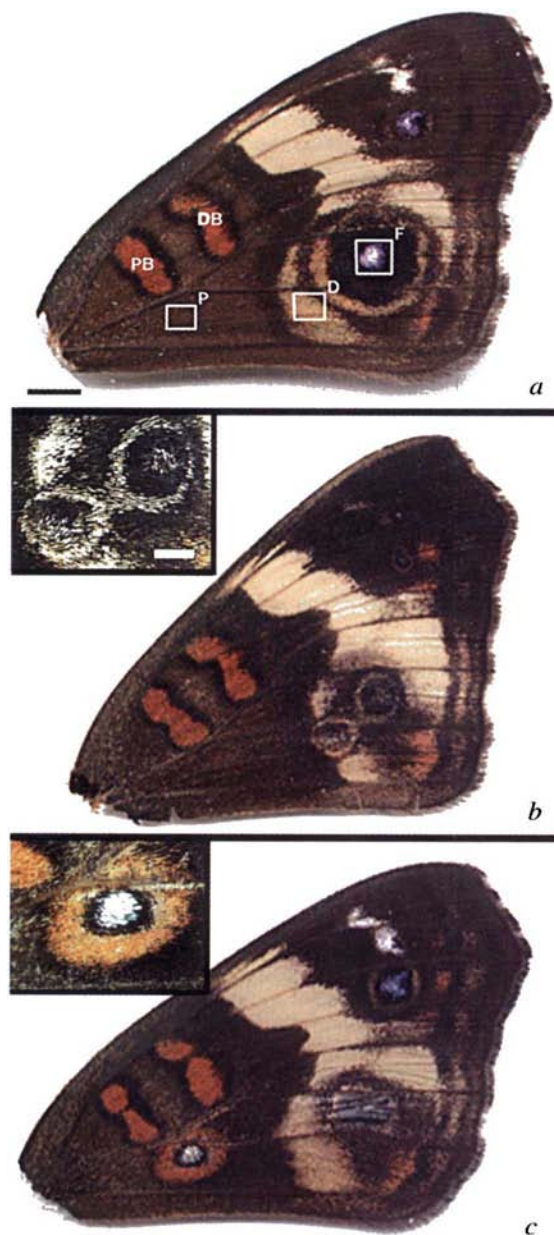


FIG. 3 The colour pattern formed by cells responding to focal signalling depends on their position. *a*, A wild-type adult *P. Coenia* forewing. The relevant pattern elements and wing positions are the posterior eyespot bearing a white focus (F) at its centre, the orange and black bands of the central symmetry system (PB and DB), the proximal site (P) posterior to the PB, and the more distal site (D) adjacent to the eyespot. *b*, *c*, The patterns formed within and around grafts of dorsal wing tissue that were transplanted 3 h after pupation to different sites within the same wing subdivision. *b*, Reciprocal grafts between the F and D sites. Transplantation of the focus to the ectopic site induces circular pigmentation (mostly buff-coloured scales) around the graft. A diminished eyespot still forms at F because the focus was removed after induction was initiated. Inset, detail. *c*, A graft from the F site to the P site induces a brilliant orange-and-black eyespot. The graft from the P site is not induced because the focus has been removed. Inset, detail.

(Fig. 3c). Control non-focal grafts had no effect when transplanted into these positions (data not shown). These results indicate that the colour pattern response to a focal signal depends on the position of the responding cells (see also ref. 16).

Different species patterns diverge early

The four stages of the eyespot developmental pathway each represent a potential point of divergence in eyespot patterns between wing subdivisions or wing surfaces within a species or between different butterfly species. We examined the dynamics of *Dll* expression to determine the point in development at which patterns diverge in several unrelated butterfly species, including some with different numbers of eyespots and others without eyespots. In *P. coenia*, a species that has two eyespots on the adult hindwing (Fig. 4b) and forewing, stripes of *Dll* expression form on the midline of each wing subdivision, just as in *B. anynana*. However, in *P. coenia* only two stripes give rise to stable foci (Fig. 4a) and eyespots, while the other stripes fade away (data not shown) and no foci or eyespots form. Thus the two eyespot-bearing species have very similar prepatterns, but diverge at the focal determination stage (stage II).

By contrast, *Danaus plexippus* (the monarch butterfly) has no adult eyespots (Fig. 4d), and no midline stripes or spots are detectable in the imaginal discs (Fig. 4c). However, the broad distal band of *Dll* expression is present, as in other butterfly wing discs and *Drosophila* wing discs¹⁷. These results suggest that, although the broad distal band of *Dll* expression is widely conserved, only species with eyespots display a larval prepatter of midline stripes of *Dll* expression, which is refined into discrete spots within specific wing subdivisions. This indicates that the development of species with eyespots diverges from those without before the larval prepatter forms (before stage I).

Seasonal polyphenism in *B. anynana*

In some butterfly species, large differences in eyespot patterns can occur between genetically identical individuals. *B. anynana* has two seasonal forms (Fig. 5a, b). The wet-season form has conspicuous ventral eyespots, submarginal 'chevron' markings, and a prominent pale medial band. These pattern elements are greatly reduced or absent in the dry-season form, which is more uniformly

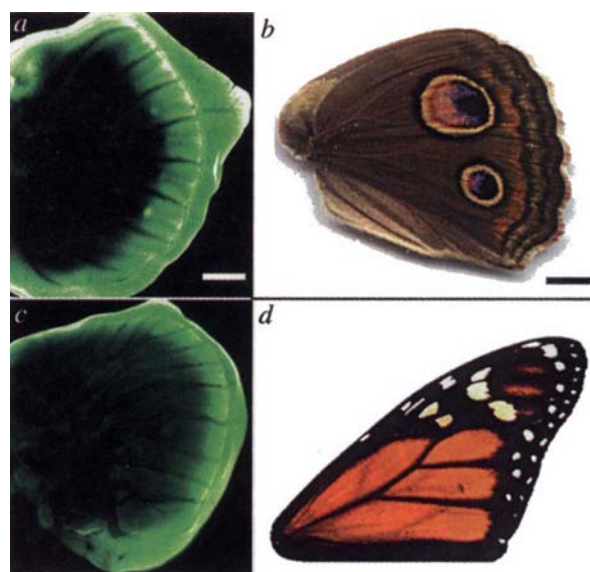


FIG. 4 Only eyespot-bearing species display a prepatter of *Dll* expression. *a*, Fifth-instar *P. coenia* hindwing imaginal disc exhibits two spots of *Dll* expression which correspond to the future position of eyespots on the adult hindwing (*b*). *c*, The fifth instar imaginal disc of *Danaus plexippus* does not exhibit any spots of *Dll* expression, but does express *Dll* in all distal cells, as in other species. *d*, The adult *D. plexippus* wing does not bear eyespots.

brown^{4,19}. This plasticity is an adaptive response to seasonal climates in Africa⁴. The dry-season butterflies rest on dead, brown leaf litter and are highly cryptic; any conspicuous markings on the exposed ventral wing surfaces can attract predatory lizards. These butterflies must persist as inactive adults for the duration of the dry season, before egg laying can begin with the rains. In contrast, eyespots are favoured in the highly active butterflies within the green herbage layer of the wet season, because they can deflect attacks by birds or lizards away from the vulnerable body. Survival analyses of marked cohorts of each form in each season have supported this hypothesis (N. Reitsma, G. H. Engelhard and P. M. Brakefield, unpublished data).

In field populations of *B. anynana* butterflies, larvae growing in hot, wet conditions develop into adults of the wet-season form, whereas the cohort of larvae growing in the transition from wet to dry season, when the temperature is declining, develop into the cryptic, dry-season butterflies^{19,20}. By rearing animals in controlled conditions in the laboratory, we have demonstrated a clear relationship between rearing temperature and wing phenotype that can be described graphically in the form of a norm of reaction²¹ (Fig. 6a). Development of the larvae at 23 °C or warmer yields the wet-season form, whereas around 17 °C produces the dry-season form. Laboratory temperature-shift experiments show that the sensitive period is the late larval stage, especially the final instar, when the initial stages of eyespot development are occurring in the wing imaginal disc²².

Genetic basis of phenotypic plasticity

There is variation within our laboratory population in the expression of phenotypic plasticity. We studied the genetic basis of variation in eyespot size by applying artificial selection on the phenotypically plastic ventral hindwing. LOW and HIGH lines were established by selecting as parents the individuals that were most similar to the dry-season form or the wet-season form, respectively, when reared at intermediate temperatures. Smooth and rapid responses to selection were observed in each line, consistent with estimates of high realized heritability from other selection experiments on eyespots^{23,24}. There were dramatic changes in wing pattern across all temperatures after 16 (HIGH) or 20 (LOW) generations (Fig. 6), leading to a divergent pair of lines with well separated 'bundles' of reaction norms for individual families. The LOW (Fig. 5c, d) and HIGH (Fig. 5e, f) selected lines now produce butterflies resembling the dry- and the wet-season forms, respectively; thus they have lost the ability to express both seasonal forms. For example, at 17 °C, HIGH-line individuals (Fig. 5e) produce eyespots similar to those of unselected individuals reared at 23 °C (Fig. 5b; the wet-season form).

To characterize the genetic basis of divergence in eyespot size and, in particular, to estimate the minimum number of genes involved, we analysed the phenotypic variance of offspring of controlled crosses of the LOW and HIGH selected lines. Wright's method²⁵ relates the difference in the means of two inbred lines to the variance of their F₂ and backcross populations. We used Lande's²⁶ modifications of Wright's method to estimate that a minimum of five or six genes contribute to the large difference in eyespot size in each sex between the selected lines (Table 1). Because of the assumptions of normal distributions, additive gene action, unlinked loci and equal allelic effects at all loci, such estimates need to be interpreted with caution^{27,28}, but deviations from the assumptions will usually cause an underestimate of the number of genes²⁸. The large sample sizes and the consistency across the estimates based on different combinations of the crosses strengthen our conclusion that the lines differ in a minimum of five or six loci.

Late regulation of phenotypic plasticity

The existence of polyphenic adult colour patterns in the wet-season and dry-season forms of *B. anynana* demonstrates that the eyespot developmental pathway is plastic and under environmen-

tal control. The divergent phenotypes of the HIGH and LOW selected lines show that the nature of this plasticity can be changed through selection to yield different genotypes specifying large or small eyespots in response to a common environment. The environmental and genetic control of the differences in eyespot size in *B. anynana* might operate at any stage; for example, focus formation could be delayed in the dry-season form, or the response to focal signalling could be diminished.

To examine how eyespot development differs between the seasonal forms and selected lines of *B. anynana*, we assayed *Dll* expression in the late larval and early pupal stages (stages II–III), because these include the period during which signalling from the focus is initiated^{15–17}. Late fifth-instar larvae display no significant divergence in area of focal *Dll* expression between the wet- and dry-season forms of the unselected stock or between HIGH and LOW lines (Fig. 7a–d; *P* is not significant by two-way analysis of variance (ANOVA)). This demonstrates that, in terms of the expression of this molecular marker, neither the environmental modulation leading to the divergence of the wet- and dry-season forms, nor the genetic differences between the HIGH and LOW lines, affect the first two stages of eyespot development.

By 24 h after pupation, however, eyespot development has clearly diverged between stock individuals reared to produce the wet- and dry-season adult forms. The areas of *Dll* expression in each focus and in the surrounding scale-forming cells are much

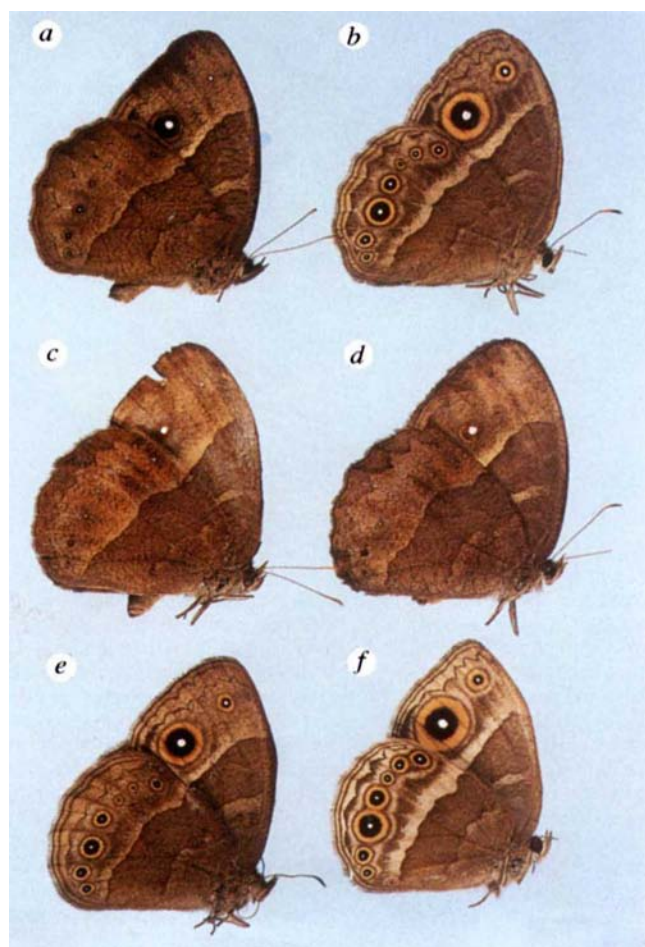


FIG. 5 *B. anynana* butterflies illustrating seasonal polyphenism in wing pattern and the response to artificial selection. All butterflies shown are representative females displaying their right ventral wings (as they would at rest). a, c, e, Reared at 17 °C; b, d, f, reared at 27 °C. Top row, dry-season form (a) and wet-season form butterflies (b) reared from the unselected stock; middle row, c and d are both LOW-line butterflies; bottom row, e and f are both HIGH-line butterflies.

larger in the wet-season form (Fig. 7e) than in the dry-season form (Fig. 7f; $P < 0.001$ by two-way ANOVA). The environmental rearing conditions must be responsible for this difference in gene expression. Similarly, HIGH-line pupae also express *Dll* in larger foci and greater numbers of surrounding scale-forming cells (Fig. 7g) than are seen in the LOW line (Fig. 7h; $P < 0.001$ by two-way ANOVA). As these lines are reared in the same environment, genetic differences must underlie these differences in *Dll* expression. Hence, by the time of signalling from the foci (stage III), environmental and genetic influences have clearly begun to affect the course of eyespot development.

The divergence in eyespot size, which is environmentally regulated in the seasonal forms and genetically controlled in the selected lines, has a common developmental basis. For *B. anynana*²⁹ and other species^{9,10,30}, the mechanisms that mediate seasonal polyphenism in response to environmental cues have

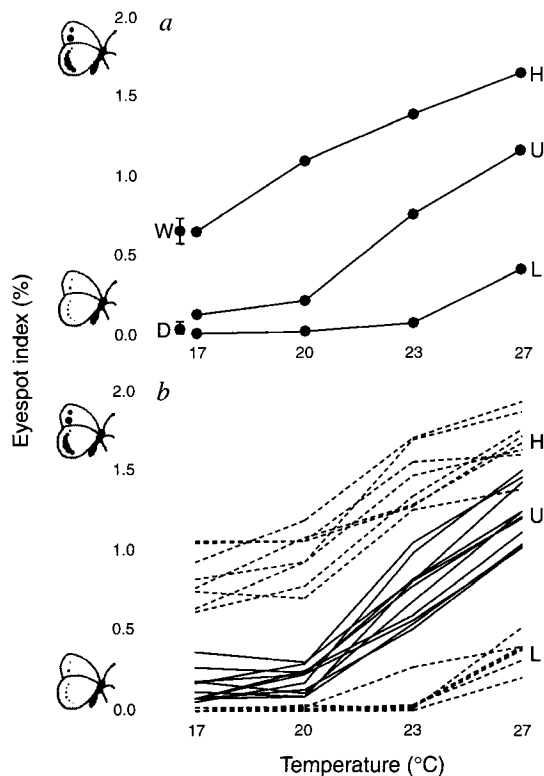


FIG. 6 Response of eyespot size to environmental temperature and artificial selection. *a*, The reaction norm (RN) underlying seasonal polyphenism in *B. anynana* and in the selected lines. Index of eyespot size is the percentage of the area of the hindwing which is covered by the black centre of the largest hindwing eyespot (spot 5). Middle line, the average RN for the unselected stock (U). Top line, RN for HIGH line (H) after selection. Bottom line, RN for LOW line after selection. The maximum value for the standard error for each data point was 0.05%, with all pairwise comparisons across lines or the stock being highly significant ($P < 0.001$). Although the direct target of selection was the size of the fifth eyespot on the ventral hindwing, analysis demonstrated that divergence occurred for all ventral eyespots, the median band and principal components describing overall plasticity in the wing pattern (see also Fig. 5). Results are consistently similar for males and females. Boxed points show eyespot index means for wild-collected butterflies of wet- (W) and dry-season (D) forms, which went through larval development at about 19°C and 23°C, respectively^{19,20}. Bars represent 95% confidence intervals. *b*, Sets of RNs for female full siblings from the low-line high-line and unselected stock, as estimated from a split-family experiment. Each RN is based on four subsamples (N is usually >4) from a single family reared at each of the four standard temperatures. Multiple ANOVA of this data shows a highly significant interaction between family and temperature for the unselected stock ($F = 2.86$; d.f. 36, 481; $P < 0.001$). This interaction between genotype and environment has been much reduced in the LOW line ($F = 0.51$; d.f. 21, 110; P is n.s.) and HIGH line ($F = 1.54$; d.f. 21, 206; P is n.s.). Similar results were obtained for males.

been shown to involve ecdysteroid hormones. The separable environmental and genetic effects on the eyespot developmental pathway indicate that: these mechanisms act to modulate the expression of regulatory genes such as *Dll*; the wild-type population must contain allelic variation for genes which influence this modulation; and selection can change allele frequencies to produce genetically divergent lines which differentially regulate the same developmental pathway and markedly transform the response to an environmental gradient.

Evolution of butterfly wing patterns

Our molecular, genetic and transplantation studies suggest that eyespot pattern is determined progressively and is regulated in four stages (Fig. 8, top). The determination of the position and number of signalling sources (foci) involves the initial deployment in the mid-fifth instar imaginal disc of regulatory genes (such as *Dll*) in a molecular prepatter within each wing subdivision (Fig. 8a). In specific subdivisions, the *Dll*-expressing foci are then

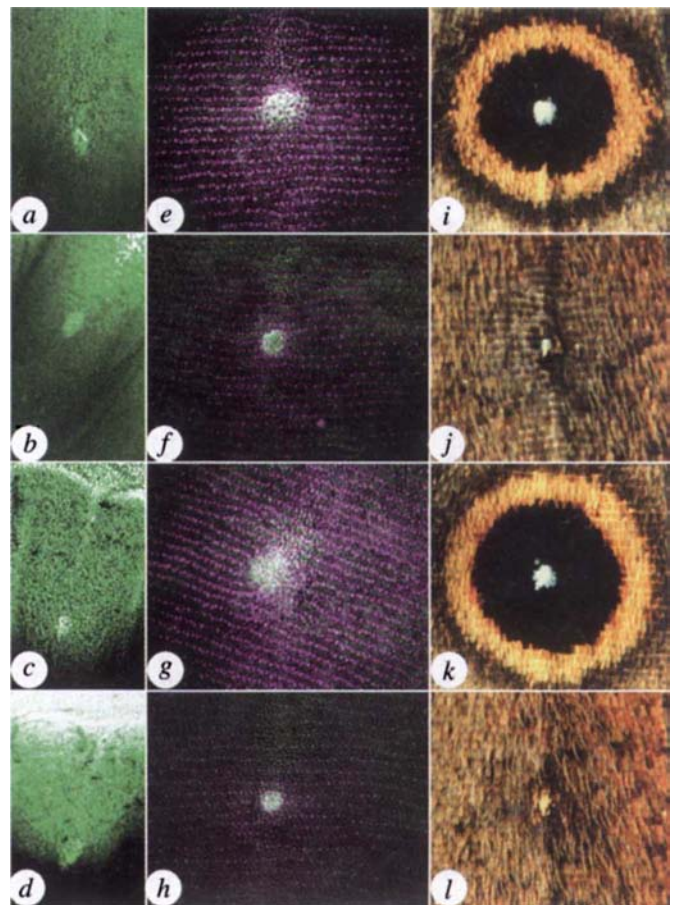


FIG. 7 Divergence in *B. anynana* eyespot development is evident by the time of signalling from the focus. *a–d*, In the late-fifth instar, *Dll* expression in wing disc epithelial cells is predictive of the locations of the eyespot centres on the adult wing. At this stage, in the ventral hindwing (representative fourth wing subdivision shown), the wet-season form (*a*) and dry-season form (*b*) of the unselected stock are similar in both the number and area of cells expressing high levels of *Dll*. *Dll* expression in the HIGH line (*c*) and LOW line (*d*) is also similar at this stage. *e–h*, By 24 h after pupation, the time of signalling from the focus, the *Dll* expression patterns have clearly diverged. The number and area of *Dll* expressing epithelial cells at the focus, and of scale-forming cells surrounding them, are greater in the wet-season form (*e*) than in the dry-season form (*f*) of the unselected stock. Similarly, the HIGH line (*g*) displays more *Dll*-expressing epithelial and scale-forming cells than the LOW line (*h*). *i–l*, Representative examples of adult fourth eyespots of the wet-season (*i*) and dry-season (*j*) forms, and the HIGH line (*k*) and LOW line (*l*).

TABLE 1 Estimates of number of genes

	Estimate 1	Estimate 2	Estimate 3	Estimate 4
Males*	6.08 ± 1.13	6.34 ± 1.17	4.81 ± 1.28	9.30 ± 2.62
Males†	9.88 ± 2.78	9.06 ± 2.09	6.41 ± 1.99	15.45 ± 6.91
Females*	10.79 ± 3.47	8.48 ± 1.91	9.74 ± 4.87	7.50 ± 1.80

Estimates of the minimum number of genes regulating the difference in fifth hindwing eyespot size between the LOW and HIGH selected lines are given with their standard errors. The values for each sex were obtained by analysis of different combinations of the parental lines and of crosses between them: estimate 1 includes F_1 hybrids and F_2 ; 2, includes LOW, HIGH, F_1 and F_2 ; 3 includes F_2 , backcrosses to LOW and to HIGH; 4 includes LOW, HIGH, F_1 and both backcrosses. See Supplementary Information for details of rearing conditions, eyespot measurement and data analysis.

* Untransformed data.

† Use of the best power transformation (0.566).

established (Fig. 8b), after which induction from the focus is mediated by signals to surrounding cells (Fig. 8c). This could involve one graded morphogen^{2,15} or a relay system involving multiple signals acting sequentially across the radius of the eyespot field¹⁶. Surrounding cells respond by eventually producing different pigmented scale types, according to the level or type of signal they receive (Fig. 8d), and their location within the wing (Fig. 3).

The great diversity of butterfly eyespot patterns reflects both the flexibility and modularity of individual stages of this developmental pathway. This flexibility is shown by both the plasticity and the rapid response to selection seen in *B. anynana*, and the pathway's modularity was dramatically illustrated by the specific effects of single-gene mutants. We have demonstrated how differences in the presence or absence (for example, *P. coenia* versus *D. plexippus*), number (*P. coenia* versus *B. anynana*), size (*B. anynana* wet- versus dry-season forms) and colour (*P. coenia* versus *B. anynana*) of eyespots can arise between species or between wing surfaces or subdivisions at discrete stages of this developmental pathway (Fig. 8; compare top and bottom panels at each stage).

The selection experiments on *B. anynana* rapidly produced dramatic differences in eyespot size which were due to genes of small phenotypic effect. The mutants described here show that genes also exist with large phenotypic effects on eyespot development (including size) that have no perceptible effect on other wing or body patterns. It is likely that the evolution of eyespot patterns in nature involves both genes with large and small effects on eyespot development. For example, in a closely related species, *B. safitza*, individuals with four forewing eyespots, very similar to the *B. anynana* Spotty mutants, occur with significant frequency in Malawi (P. M. Brakefield, unpublished data). These observations suggest that the regulation of the eyespot developmental pathway is such that eyespot patterns can evolve rapidly and independently of other wing-pattern elements and body structures. Eyespots respect positional coordinates that are established by a more general regulatory mechanism in that they are always centred in the midline between wing veins (and *Cyclops* demonstrates that

disruption of these coordinates can perturb eyespot formation). However, the proximodistal position, number, size and colour of eyespots seems to be largely uncoupled from other patterns and structures, and can evolve rapidly through a relatively small number of changes in regulatory gene interactions.

These results in butterflies may have general implications for the evolution of colour patterns in animals. The diversity of colour patterns in other speciose taxa, such as fish, snakes, birds and various insect orders, may involve analogous developmental pathways that are uncoupled from those that control the formation of body structures. Indeed, single or small numbers of loci control dramatic features of colour pattern in snakes^{31,32}, land snails³³ and fish³⁴. The ability to manipulate eyespot patterns makes butterflies more accessible experimentally, but it will be important to learn how colour patterns in other taxa develop and evolve. □

Methods

Antibody production and immunohistochemistry. Antibodies were raised and affinity purified against the 198 amino-acid N-terminal portion of the *P. coenia* DII protein expressed in *Escherichia coli*. Imaginal discs were fixed in 0.1 M PIPES, pH 6.9, containing 1 mM EGTA, 2 mM $MgSO_4$, 1% Triton X-100, and 5 mg ml⁻¹ bovine serum albumin (BSA) for 1–2 h at 4 °C and incubated with primary antibody for 24–48 h at 4 °C. The discs were then washed four times for 20 min each at 4 °C, incubated for 2 h at 4 °C with fluorescein-conjugated secondary antibody, washed and incubated overnight, then mounted in 0.1 M Tris, pH 8.8, containing 10% glycerol. Images were gathered on an MRC 600 laser scanning confocal microscope.

Focal transplants. All manipulations were performed on 3-hour-old *P. coenia* pupae that were cultivated with a regular light–dark cycle. Landmarks for the foci present on the overlying pigmented pupal cuticle¹⁵ and pupal tracheae were used to guide the excision and transplantation of small squares of tissue. The donor grafts were rotated 90° to identify donor from host tissue; this changes the scale orientation on tissue versus the surrounding host scales.

***Bicyclus* husbandry and selection for eyespot size.** The stock was established from more than 80 gravid females collected in 1988 from near Nkhata Bay in Malawi. It is maintained at an adult population size of 600–800 with some overlap of generations. Effective population size is one half of census number. All *Bicyclus* individuals were reared under a 12 h:12 h light:dark cycle. Wet-season forms of the stock were reared at 27 °C, dry-season forms at 17 °C.

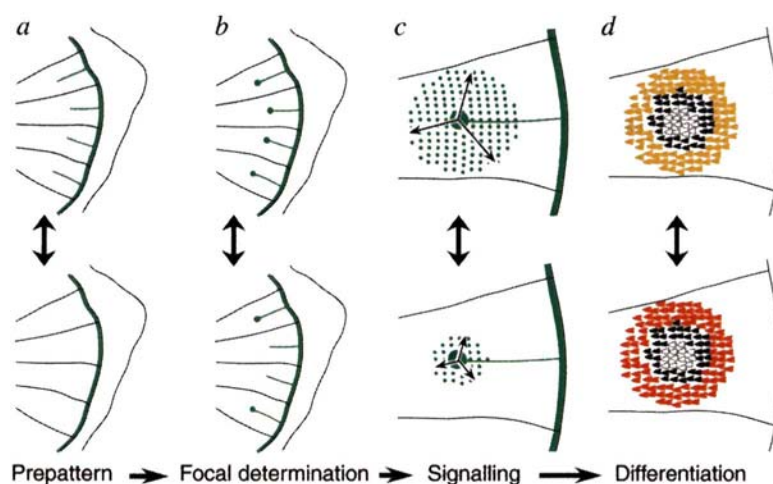


FIG. 8 Regulation of eyespot formation and diversity. Eyespot formation and diversity is regulated in four stages. Note the progressive specification of the eyespot pattern and the differences that arise in this developmental pathway between species (contrast top and bottom rows). a, In the larva, a prepattern of gene activity creates the potential focal pattern reflected by early *DII* expression, which occurs only in eyespot-bearing species. b, Foci are determined and *DII* expression is stabilized only in specific wing subdivisions; the number of foci differs between wing surfaces and butterfly species. c, In the pupa, signalling from the focus (green circle) induces surrounding cells; different sized eyespots are controlled by the size of the focus. d, Induced cells later differentiate into scales of different colours depending on their distance from the focus and their position in the wing.

LOW and HIGH lines were raised at 20 °C except during selection. Truncation selection was applied in each line after measurement of wing length and eyespot diameter. Selection for the first ten generations was applied to butterflies reared at 20 °C. Because of a reduction in phenotypic variation, selection was then continued by rearing the LOW line at 23 °C for ten generations, and the HIGH line at 18 °C for six generations. We measured 300–400 butterflies of each sex in each generation. At least 40 females were used as selected parents in each line after being held in a mating cage with about 100 of the most extreme males. Reaction norms were estimated using replicated subsamples of eggs reared at the four temperatures (other conditions were maintained as standard). Wing characters were measured with high repeatability using an image analysis system.

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- Cott, H. B. *Adaptive Coloration in Animals* (Methuen, London, 1940).
- Endler, J. A. *Natural Selection in the Wild* (Princeton Univ. Press, 1986).
- Nijhout, H. F. *The Development and Evolution of Butterfly Wing Patterns* (Smithsonian Inst. Press, Washington, 1991).
- Brakefield, P. M. & Larsen, T. B. *Biol. J. Linn. Soc.* **22**, 1–12 (1984).
- Brakefield, P. M. & Reitsma, N. *Ecol. Entomol.* **16**, 291–303 (1991).
- Shapiro, A. M. *Evol. Biol.* **9**, 259–333 (1976).
- Kingsolver, J. G. *Evolution* **49**, 932–941 (1995).
- Kingsolver, J. G. *Evolution* **49**, 942–954 (1995).
- Rountree, D. B. & Nijhout, H. F. *J. Insect Physiol.* **41**, 987–992 (1995).
- Rountree, D. B. & Nijhout, H. F. *J. Insect Physiol.* **41**, 1141–1145 (1995).
- Stearns, S. C. *Bioscience* **39**, 436–445 (1989).
- Via, S. et al. *Trends Ecol. Evol.* **10**, 212–217 (1995).
- Schlichting, C. D. & Pigliucci, M. *Evol. Ecol.* **9**, 154–168 (1995).
- Pigliucci, M. *Trends Ecol. Evol.* **11**, 168–173 (1996).
- Nijhout, H. F. *Dev. Biol.* **80**, 276–274 (1980).
- French, V. & Brakefield, P. M. *Dev. Biol.* **168**, 112–123 (1995).
- Carroll, S. B. et al. *Science* **265**, 109–114 (1994).
- Brakefield, P. M. & French, V. *Acta Biotheor.* **41**, 447–468 (1993).
- Windig, J. J., Brakefield, P. M., Reitsma, N. & Wilson, J. G. M. *Ecol. Entomol.* **19**, 285–298 (1994).
- Brakefield, P. M. & Mazzotta, V. J. *Evol. Biol.* **8**, 559–573 (1995).
- van Noordwijk, A. J. *Bioscience* **39**, 453–458 (1989).
- Kooi, R. E., Brakefield, P. M. & Schlattman, E. G. M. *Proc. Exp. Appl. Entomol.* **5**, 47–52 (1994).
- Holloway, G. J., Brakefield, P. M. & Kofman, S. *Heredity* **70**, 179–186 (1992).

Crosses to estimate gene number. The black diameter of the fifth eyespot (corrected for wing length) was measured with a binocular microscope fitted with a micrometer. All reciprocal crosses were made and reared separately. Crosses with pooled sample sizes in parentheses (male:female) were: parental LOW (123:113) and HIGH (105:137); F_2 (206:209); F_2 (302:284); backcrosses to LOW (516:444), and to HIGH (607:581). Several crosses in females show departures from normality which are resistant to data transformation. Two crosses in males also depart from normality, but application of Taylor's power law reduces this to a single cross, the F_2 .

For further experimental methods and statistical analyses, see Supplementary Information.

- Monteiro, A. F., Brakefield, P. M. & French, V. *Evolution* **48**, 1147–1157 (1994).
- Wright, S. *Evolution and the Genetics of Populations* Vol. 1, *Genetics and Biometrical Foundations* (Univ. Chicago Press, 1968).
- Lande, R. *Genetics* **99**, 541–553 (1981).
- Cockerham, C. C. *Genetics* **114**, 659–664 (1986).
- Zeng, Z.-B., Houle, D. & Cockerham, C. C. *Genetics* **126**, 235–247 (1990).
- Koch, P. B., Brakefield, P. M. & Kesbeke, F. J. *Insect Physiol.* **42**, 223–230 (1996).
- Nijhout, H. F. *Insect Hormones* (Princeton Univ. Press, 1994).
- Zweifel, R. G. J. *Heredity* **72**, 238–244 (1981).
- King, R. B. *Can. J. Zool.* **71**, 1985–1990 (1981).
- Sheppard, P. M. *Natural Selection and Heredity* (Harper, New York, 1960).
- Endler, J. A. *Evol. Biol.* **11**, 319–364 (1978).

SUPPLEMENTARY INFORMATION is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from Mary Sheehan at the London editorial office of Nature.

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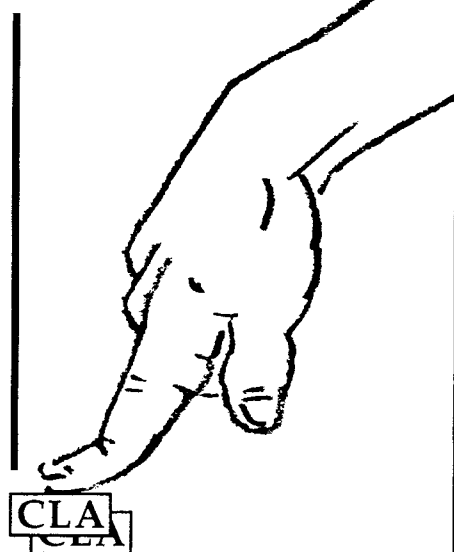
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Efficient detection of brown dwarfs using methane-band imaging

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BROWN dwarfs lie in the mass range between the most massive Jupiter-like planets and the least massive stars. They are much less luminous than stars, and so may provide a fraction of the baryonic dark matter in our Galaxy. Only one unambiguous detection of a brown dwarf has been made to date¹⁻⁶—Gl229B, a low-mass companion to the nearby star Gl229A. The detection⁴ of strong methane-band absorption in the spectrum of Gl229B, a feature restricted to cool substellar objects⁵⁻⁹, lends weight to the idea⁷ that differential methane-band imaging (the subtraction of an image taken in the methane band from a continuum-light image taken in the same spectral region) should provide an efficient method for detecting brown dwarfs. Here we demonstrate the potential of this approach by obtaining an image of Gl229B with less than two minutes of integration time. This technique promises efficient detection of both isolated brown dwarfs in crowded regions, and brown dwarfs orbiting close to their primary stars.

Non-stellar objects, including shells and disks around stars and background galaxies, are very unlikely to exhibit a Gl229B-like methane-band spectral signature because the combination of densities and temperatures in these objects will not allow a substantial quantity of methane to form^{5,6,8,9}. Further, the depth and breadth of the Gl229B methane absorption is unlike that due to any other known atomic or molecular feature in the near-infrared spectral region (Fig. 1).

Isolated (field) brown dwarfs are very difficult to identify owing to their low luminosity, leading to confusion with background objects¹⁰⁻¹⁴. Differential methane-band imaging will enable the identification of field brown dwarfs in an efficient manner because only methane-rich objects will remain in the difference image. In contrast, searching for brown dwarfs orbiting nearby stars ('superplanets') is hindered by the large flux contrast between the two and their likely small angular separation. Because stars are essentially flat¹⁵ in the methane-band spectral regions, differential imaging maximizes the contrast ratio between the star and superplanet by effectively removing the stellar point-spread function while leaving the superplanet image nearly unaltered. Because brown dwarfs emit very little energy at optical wavelengths, and sensitivity above a wavelength of 2.5 μm is dramatically reduced using ground-based telescopes, the 1–2.5 μm spectral region is best for brown-dwarf detection at present.

As proof of concept, observations of Gl229AB were obtained on 25 April 1996 UT as part of the regular Service Observing Program at the NASA Infrared Telescope Facility (IRTF). The data were obtained using the 256 $\times$ 256 InSb camera NSFCAM coupled with an $R \equiv \lambda/\Delta\lambda = 90$ circular variable filter (CVF) and a 6-arcsec-diameter unapodized cold focal plane occulting mask. The conditions were clear and dark with no moon present. The Gl229AB images were obtained at airmasses ranging from 2.3 to 2.4 in the H-band

region and 2.4 to 2.6 in the K-band region. Four 10-s exposures were obtained in the H band at 1.58 μm (continuum) and at 1.72 μm (methane band), and in the K-band four 10-s exposures at 2.09 μm (continuum) and one 10-s exposure at 2.27 μm (methane band) were obtained. The wings of the point-spread function of Gl229A extend ~ 15 arcsec radially outwards before reaching the background noise level.

The images were reduced by simply producing object minus sky frames. No flat fields were taken. The Gl229AB frames were registered by elliptical fitting to the annulus of the Gl229A image visible outside the mask, and were then co-added at each wavelength. The resulting summed methane-band images were scaled and subtracted from the corresponding continuum images to produce difference images at both H (Fig. 2) and K.

Differential imaging at H yielded a 7σ Gl229B detection (Fig. 2c), 29σ when smoothed (Fig. 2d). Gl229B appears at the expected position³ of $\Delta\text{RA} = +2.4$ arcsec, $\Delta\text{dec.} = -7.3$ arcsec. No additional companions were detected to $H \approx 15$ mag (3σ) within 4 arcsec (23 AU) of Gl229A (the mask edge). Differential imaging at K band worked just as well as at H, but the Gl229B detection is only marginally above the noise level at K due to the lower flux of Gl229B, by a factor^{1,2} of ~ 4 , for our CVF bandpasses (Fig. 1). The relative strengths of the H- and K-band methane absorption can vary for brown dwarfs with different temperatures and near-infrared spectra^{5,6}. The continuum minus methane-band differential technique effectively removes systematic effects, including the asymmetric point-spread function of Gl229A (an M1 V star). Continuum and methane-band exposures are taken in fairly rapid succession so that no systematics are introduced due to substantial airmass changes or deformation of the telescope primary mirror. With the exception of the inner regions of the primary star image, which were affected by the movement of the mask relative to the star, the subtraction effectively removed the primary star point-spread function, allowing the identification of Gl229B in both the H and K differential images. Ground-based seeing scales as $\lambda^{-1/2}$, compared to λ^{-1} for diffraction-limited imaging, so no corrections to the point-spread functions due to the small wavelength differences between the images were required.

Differential methane-band imaging is far more efficient than other direct-imaging brown-dwarf search strategies, such as broad-

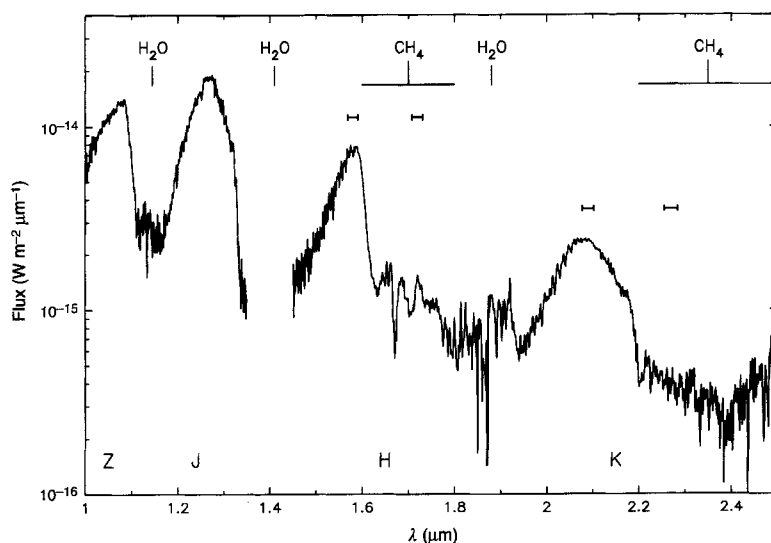


FIG. 1 The medium-resolution near-infrared spectrum of Gl229B (from ref. 1). The absorption bands due to methane and water vapour are indicated. The bandwidths (full-width at half-maximum) of the CVF settings used in the observations are shown by horizontal bars in the H and K spectral regions. Broadband magnitudes for Gl229B are $J = 14.2$, $H = 14.3$ and $K = 14.4$ (ref. 2).

band photometric imaging in the J, H and K bands. In only a few minutes observing time at the IRTF, using a state-of-the-art infrared camera and tunable filter, it is possible to detect Gl229B-like brown dwarfs in the vicinity of nearby stars at the 10σ level. Such a differential imaging technique using relatively broad ($R \approx 10$) filters—either custom-made or synthetic; for instance, a combination of K and K_{short} filters to isolate the K-band methane and continuum features—and using either an apodizing, coronagraphic system or direct imaging, appears to give the best chance for rapidly detecting Gl229B-like brown dwarfs in the vicinity of nearby stars as well as in the field. Simultaneous imaging in the brown-dwarf continuum and methane-band regions will eliminate ground-based seeing variations, and an adaptive optics/tip-tilt system will greatly help with image registration. It is natural to consider extending such a survey to the Hubble Space Telescope (for the best possible spatial resolution, including complementary observations of the near-infrared H_2O bands masked by the Earth's atmosphere). This method could also be used for similar types of studies, such as searches for brown dwarfs in gaps in circumstellar disks like that around β Pictoris or wherever they sufficiently dominate the infrared light. In the future, this differential imaging concept could be extended to other wavelengths, in particular to the thermal infrared for observations of other absorption bands such as the $15\text{-}\mu\text{m}$ CO_2 band and the $9\text{-}\mu\text{m}$ ozone

band^{16–18}, possible characteristics of the atmospheres of terrestrial-class planets. □

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1. Geballe, T. R., Kulkarni, S. R., Woodward, C. E. & Sloan, G. C. *Astrophys. J.* **467**, L101–L104 (1996).
2. Matthews, K., Nakajima, T., Kulkarni, S. R. & Oppenheimer, B. R. *Astron. J.* (in the press).
3. Nakajima, T. et al. *Nature* **378**, 463–465 (1995).
4. Oppenheimer, B. R., Kulkarni, S. R., Matthews, K. & Nakajima, T. *Science* **270**, 1478–1479 (1995).
5. Allard, F., Hauschildt, P. H., Baraffe, I. & Chabrier, G. *Astrophys. J.* **465**, L123–L127 (1996).
6. Marley, M. S. et al. *Science* **272**, 1919–1921 (1996).
7. Smith, W. H. *Publ. Astron. Soc. Pacif.* **99**, 1344–1353 (1987).
8. Tsuji, T. *Annu. Tokyo Astron. Observatory Ser. II* **9**, 1 (1964).
9. Tsuji, T., Ohnaka, K., Aoki, W. & Nakajima, T. *Astron. Astrophys.* **308**, 29–32 (1996).
10. Alcock, C. et al. *Astrophys. J.* **461**, 84–103 (1996).
11. Flynn, C., Gould, A. & Bahcall, J. N. *Astrophys. J.* **466**, L55–L58 (1996).
12. Bahcall, J. N., Flynn, C., Gould, A. & Kirhakos, S. *Astrophys. J.* **435**, L51–L54 (1994).
13. Kuijken, K. & Gilmore, G. *Mon. Not. R. Astron. Soc.* **239**, 651–664 (1989).
14. Bahcall, J. N. *Annu. Rev. Astron. Astrophys.* **24**, 577–611 (1986).
15. Jones, H. R. A., Longmore, A. J., Jameson, R. F. & Mountain, C. M. *Mon. Not. R. Astron. Soc.* **267**, 413–423 (1994).
16. Angel, J. R. P., Cheng, A. Y. S. & Woolf, N. J. *Nature* **322**, 341–343 (1986).
17. Burke, B. F. *Nature* **322**, 340–341 (1986).
18. Owen, T. *Strategies for the Search for Life in the Universe* (ed. Papagiannis, M. D.) 177–185 (Reidel, Dordrecht, 1980).

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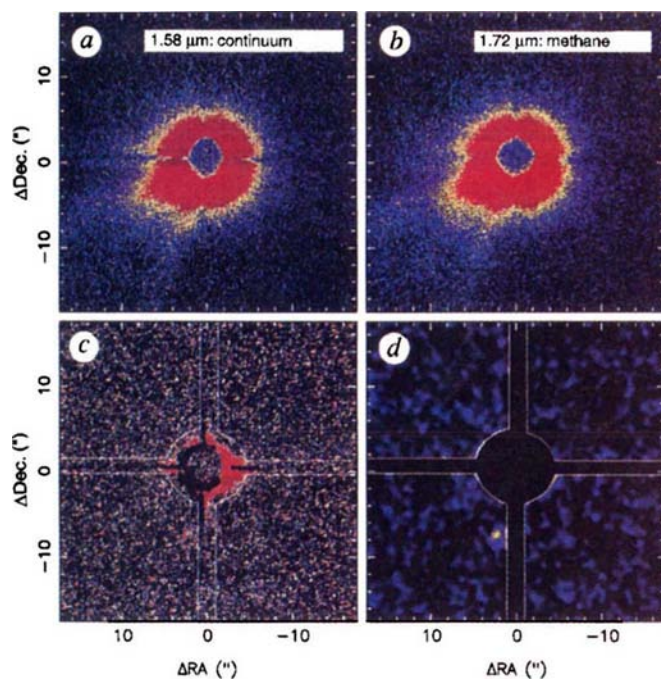


FIG. 2 Differential methane-band imaging in the H band. The plate-scale used was 0.15 arcsec per pixel giving a field of view of ~ 36 arcsec on a side after image registration (1 arcsec = 5.7 au at the Gl229AB distance). The seeing, estimated from Gl229B in the difference image and from a photometric standard observed both before and after the Gl229B observations, is ~ 0.8 arcsec FWHM. North is up and East is to the left. *a*, Sum of four 10-s sky-subtracted $R = 90$ CVF images taken at the continuum wavelength of $1.58\text{ }\mu\text{m}$. *b*, As *a*, but for the methane-band absorption wavelength at $1.72\text{ }\mu\text{m}$. *c*, The continuum minus methane-band difference image. The solid white lines delineate the region affected by the occulting mask and its support struts, including drift motion. No image stabilization was used—small tracking errors allowed the image of Gl229A to drift by up to 2 arcsec relative to the fixed occulting mask between successive frames, with most of the motion in right ascension. This image is ~ 3 times the stretch of *a* and *b*. *d*, As *c*, but after smoothing with a 0.75-arcsec FWHM gaussian in order to enhance Gl229B. The brown dwarf Gl229B is clearly revealed in the difference image, despite being hidden in the asymmetric wings of the Gl229A point-spread function shown in *a* and *b*. The region affected by the occulting mask and struts was ignored during smoothing and deleted from *d*.

Thermally stable nonlinear optical activity in a smectic-A liquid crystal

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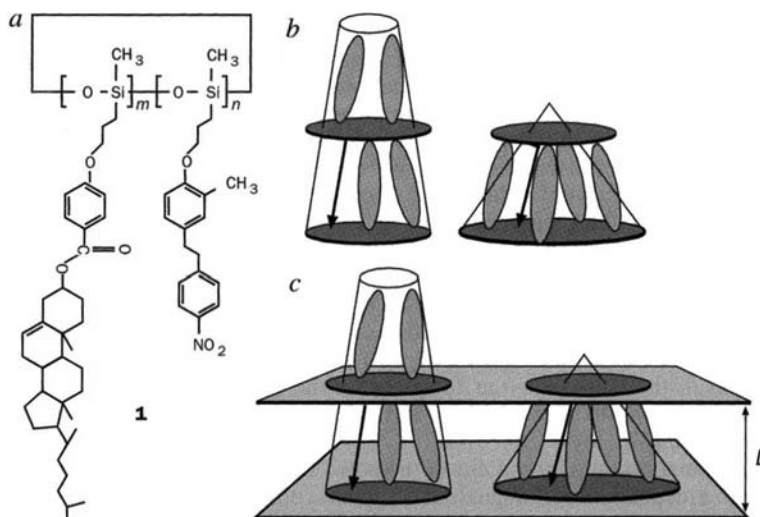
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THE key requirement for a material to exhibit nonlinear optical (NLO) activity is the presence of non-centrosymmetric (polar) order, an attribute that is usually restricted to certain crystalline classes and ferroelectric liquid crystals^{1,2}. NLO activity can also be obtained in some amorphous organic materials by applying an intense electric field (corona discharge) above the glass transition temperature, T_g , and subsequently quenching the field-induced polar orientation order^{3,4}. Such materials are attractive for NLO device applications, as they promise lower costs and easier processability than their crystalline organic and inorganic counterparts⁵. But field-induced polar order is not stable, and the eventual return to equilibrium (apolar) order results in a deterioration of NLO activity, particularly at temperatures near T_g (refs 6, 7). Here we show that this thermally activated decay of polar order can be circumvented by using a liquid crystal in which both mesogens (molecules that induce a liquid-crystal phase) and NLO-active chromophores are appended to macro-

FIG. 1 a, The cyclic macromolecule **1** is composed of randomly attached side chains of the mesogen cholesteryl-4-allyloxybenzoate (80 mol%) and the NLO-active chromophore 3-methyl-4-allyloxy-4'-nitrostilbene (20 mol%), that is, $\langle n \rangle = 4$, $\langle m \rangle = 1$. The chromophore was prepared by reacting allyl bromide with 4-hydroxy-3-methyl-nitrostilbene (ref. 23). Cholesteryl-4-allyloxy benzoate was obtained by reacting allyloxybenzoic acid with cholesterol under mild esterification conditions²⁴. Attachment of the side chains to the cyclic siloxane was performed through standard hydrosilation chemistry employing a dicyclopentadienylplatinum(II) chloride catalyst²⁵. The hydrosilation reaction progress was followed by monitoring the disappearance of the Si-H stretch at $2,160\text{ cm}^{-1}$ using Fourier transform infrared spectroscopy and the product was purified by repeated precipitation from toluene. ^1H NMR and thin-layer chromatography indicated no residual alkene present in the product. b, Schematic illustration of two of a multiplicity of regiochemical substitution patterns in macromolecule **1**. The shaded ellipsoid represents the steroidal mesogen and the arrow stands for the hyperpolarizable nitrostilbene chromophore. This illustration depicts the cone-like tertiary conformations that **1** could adopt. c, The conical conformations of **1**, shown embedded in a lamellar suprastructure with a layer spacing L .



molecular siloxane rings. We find that a shear-aligned melt of these composite macromolecules gives rise to a material with a monodomain lamellar superstructure that retains bistable, field-induced polar order above T_g . We attribute the thermal stability of these materials to an energetically favoured polar packing arrangement of the constituent macromolecules.

Although transparent monodomains of low-molar-mass liquid crystals (LCs) can be readily fabricated using a combination of shear and substrate surface treatments, LCs rarely form stable glasses. Consequently, LC fluid phases must be contained and therefore, with the exception of inherently non-centrosymmetric LCs^{2,8}, have limited utility in NLO applications. High-molar-mass polymeric LCs do form glasses, but apart from elastic networks comprised of ferroelectric phases⁹, it is difficult to produce defect-free textures in the very viscous melts. These limitations can be obviated with macromolecular cyclic siloxane LCs^{10–12}: excellent alignment and glass-forming properties are exhibited by the NLO-active cyclic siloxane co-oligomer (compound **1**; Fig. 1). Unlike its linear copolymer analogue which has a glass transition below room temperature, the steric crowding in **1** and variable regiochemical substitution patterns on the siloxane ring impose packing constraints (Fig. 1b, c) that increase the glass transition temperature of the cyclic macromolecule ($T_g = 63^\circ\text{C}$). The smectic-A (S_A) mesophase of **1** persists to $\sim 225^\circ\text{C}$ where it transforms to a cholesteric texture with a clearing point of $\sim 240^\circ\text{C}$. The glass transition in **1** was confirmed using differential scanning calorimetry and thermal mechanical analysis for both bulk and shear-aligned E-field-poled films.

Transparent, defect-free monodomain glass films ($\sim 10\text{ }\mu\text{m}$ thick) can be fabricated from the highly scattering, focal conic, S_A mesophase of **1** by shearing at $\sim 200^\circ\text{C}$ and quenching to room temperature. The homeotropic geometry (smectic layers tangent to a glass substrate that had been coated with indium tin oxide) in the monodomain glass is confirmed by its X-ray reflectivity (Fig. 2)¹³. The steroidal mesogens and chromophores stratify into lamellae with their major axes normal to layers (Fig. 1c). Additionally, as the siloxane component is chemically very distinct from the hydrocarbon-based steroid and chromophore side chains, the lamellar structure is further stabilized by nanophase separation of the siloxane component from the hydrocarbon part¹¹. The resulting periodic variation in the scattering density yields a smectic layer spacing $L \approx 28\text{ }\text{\AA}$, approximately the length of the fully extended cholesterol mesogen and its linkage to the siloxane ring. The high-resolution X-ray reflectivity shows that the smectic reflection can be deconvolved into two components (Fig. 2b and c) that are differentially perturbed by E-field poling. This

subtle structural transformation in an organic NLO material induced by E-field poling is evidence for the E-field-induced supramolecular reorganization proposed by Wang *et al.*¹⁴. The transformation is reversible only by shearing the mesophase or by prolonged annealing at elevated temperature.

The E-field-induced polar order in films of **1** was characterized by measurements of second-harmonic generation (SHG) employing unfocused, horizontal-polarized light from a Nd:YAG laser

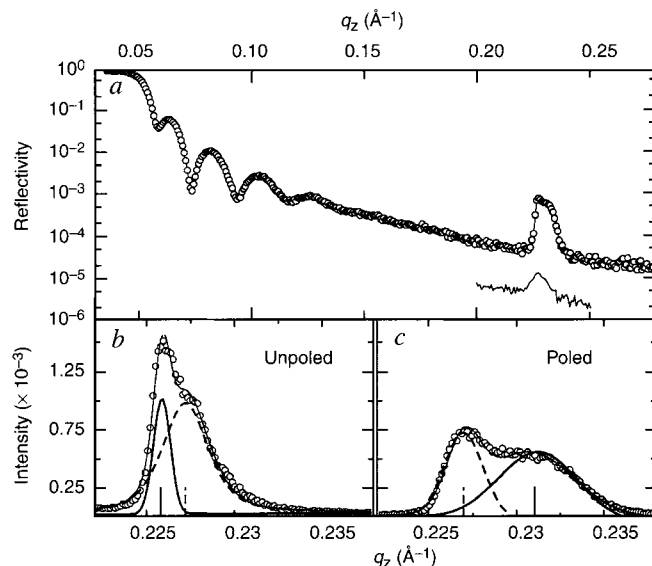


FIG. 2 a, X-ray reflectivity versus q_z , the scattering vector normal to the film, exhibited by a homeotropic ($10\text{ }\mu\text{m}$ -thick) shear-aligned film of the cyclic macromolecule **1** (data points); the Kiessig fringes at $q_z \approx 0.05\text{--}0.10\text{ }\text{\AA}^{-1}$ are due to the $\sim 275\text{-}\text{\AA}$ indium tin oxide coating on the glass substrate and the reflection at $q_z \approx 0.23\text{ }\text{\AA}^{-1}$ arises from the smectic layer spacing. For the latter, the solid line is the reflected intensity (shifted down by one decade for clarity) from a spin-coated sample showing negligible smectic layering parallel to the substrate. b, c, Reflected X-ray intensity q_z for: b, unpoled, shear-aligned smectic film (data points); c, E-field-poled, shear aligned smectic film ($\sim 8\text{ kV}$ at $9\text{ }\mu\text{A}$ for 10 min at 70°C (data points). Both poled and unpoled samples were subjected to identical shear-quench-anneal (at 70°C for 10 min) procedures before being examined by X-ray reflectivity. The dashed and solid lines represent Lorentzian fits to the lineshapes and yield spacings of 27.8 and $27.7\text{ }\text{\AA}$ (unpoled film) and 27.7 and $27.2\text{ }\text{\AA}$ (poled film) respectively.

(1,064 nm wavelength; 10-ns pulse duration, 0.1 mJ per pulse, Q-switched at 10 Hz). A reference channel, (100)-oriented quartz, and a sample channel containing the film on the heating/poling stage were ratioed (sample: reference) to give a SHG signal, I_{SHG} , that was independent of energy fluctuations in the laser output. The NLO coefficients d_{33} and d_{31} were measured by the Maker-Fringe method and the film thickness and the refractive indices were measured by a Metricon M-line waveguide. Ultraviolet/visible measurements of **1** show no absorption at 532 nm, that is, non-resonant SHG phenomena dominate. Although modest by contemporary standards¹⁵, the observed values of the second-order NLO coefficients, $d_{33} = 10 \text{ pm V}^{-1}$ and $d_{31} = 0.4 \text{ pm V}^{-1}$, are comparable to values for inorganic crystals even though the chromophore content of **1** is only 20 mol%. The relative magnitudes of d_{33} and d_{31} are consistent with the general structural features of the S_A phase—the ‘long axis’ of the chromophore is oriented normal to the lamellae (and substrate) in the homeotropic film (Fig. 1c)

The temporal/thermal stability of the poled LC films is striking. The time-dependent SHG signal $d_{33}(t)$ appears coincidentally with

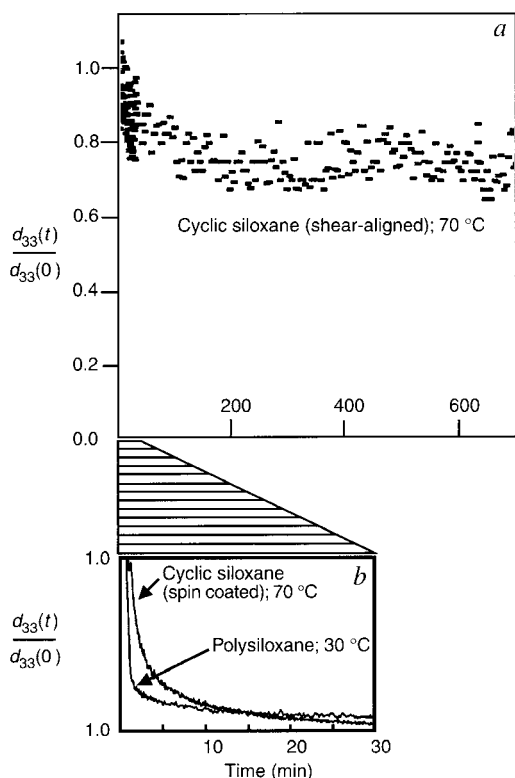


FIG. 3 Temporal evolution of the generated second harmonic signal; a, shear-oriented homeotropic film of **1** where the decay of E-field-induced polar orientational order at 70 °C after removal of the field ($t = 0$) is shown in the form of the ratio $d_{33}(t)/d_{33}(0)$ versus t . The film was prepared by: first, shearing the smectic melt between transparent substrates at elevated temperature (under the polarizing microscope to confirm the production of a uniformly oriented monodomain); second, vitrifying the aligned homeotropic monodomain; third, subsequent removal of one substrate to expose the sample to the corona E-field. Glass coated with indium tin oxide was used as the bottom substrate (the eventual ground electrode in the corona poling step) and an optically transparent (compression-mounded KBr) ‘infrared pellet’ was used as the top shear surface as it could be readily removed from the shear-aligned S_A glass by dissolution in water. (After washing with deionized water for 30 min, the film was dried in a vacuum oven for 24 h at 40 °C.) Such films are optically transparent and show slight residual (strain-induced) birefringence under crossed polars. The residual birefringence disappears after annealing (65 °C for 10 min) suggesting the formation of a macroscopically-oriented homeotropic film. b, Relaxation of $d_{33}(t)/d_{33}(0)$ for a spin-coated film of **1** at 70 °C, and a shear-aligned (non-homeotropic) film of the linear polymer analogue of **1** at 30 °C ($\sim 10^\circ$ above its T_g).

the application of the E-field above T_g (–8 kV and 9 μA corona). However, contrary to all previous reports^{16–18}, the SHG signal persists above the glass transition temperature in the absence of the poling field. Figure 3a shows $d_{33}(t)/d_{33}(t = 0)$ versus time after removal of the E-field (at $t = 0$) at 70 °C, 7° above T_g . After an initial decay ($\sim 15\%$), the SHG intensity remained essentially unchanged on a timescale of tens of hours; the I_{SHG} half-life is estimated to be $\sim 10^6$ s at 90 °C. Additionally, the E-field-induced polar order is bistable: the sign of the polarity can be switched and the SHG intensity can be modulated by applying a second E-field to the free surface of a previously poled smectic film, that is, the polar sense ‘flips’ through an angle of 180° and correspondingly I_{SHG} passes through zero in a matter of seconds before returning to its initial intensity.

In conventional amorphous polymers, a rapid decay of I_{SHG} with a half-life ≤ 100 s is always observed near T_g ^{7,16–18}. We observe a similar rapid decay of I_{SHG} for the linear siloxane copolymer analogue of **1** (that fails to form a macroscopically aligned homeotropic texture) and in a film of **1** prepared by spin coating (Fig. 2b). However, the latter observation is not disconcerting as the morphology of the spin-coated film of **1** is very different from the shear-aligned film: X-ray reflectivity shows a negligible fraction of S_A layers oriented parallel to the substrate (homeotropic texture) in the spin-coated film, as evidenced by a virtual absence of the 001 reflection (subordinate q_z scan in Fig. 2a). Thus the robust E-field-induced polar order exhibited by **1** is intimately related to the interplay between the cyclic siloxane’s overall shape (relative to the linear polymer) and intermolecular packing preferences associated with a well developed lamellar supramolecular structure formed by the shear-alignment process. At a sufficiently high temperature (for example, $T = 90^\circ\text{C}$), I_{SHG} will slowly decay, but even 30° above T_g the stability of the polar order in shear-aligned films of **1** is remarkable: the I_{SHG} half-life in **1** is $\sim 10^5$ s at 90 °C, three orders of magnitude larger than half-lives exhibited by typical amorphous polymers below T_g (refs 7, 16–18).

The observed thermal stability of the SHG and the bistability of the E-field-induced polar order are reminiscent of phenomena found in ferroelectric liquid crystals. Although the steroidal mesogenic side chain in **1** is indeed chiral, there is no evidence from X-ray diffraction or optical microscopy for a chiral S_C phase before or after poling. A possible explanation of the unusual NLO behaviour that is consistent with all of our observations may derive from the global shapes of **1**: the regiochemistry and steric crowd-

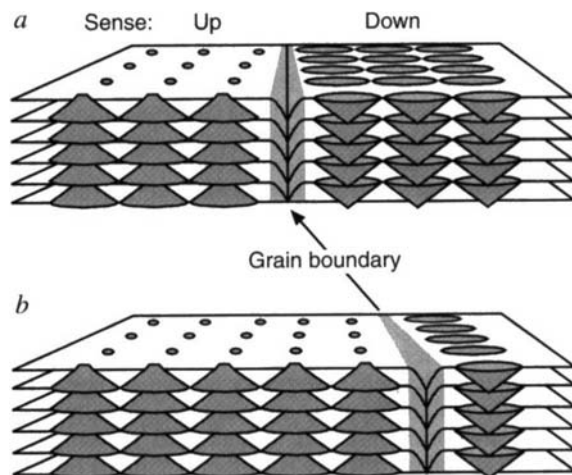


FIG. 4 Schematic illustration of the packing of idealized conical macromolecules in distinct regions with opposite polarity (‘up’ and ‘down’ respectively) separated by a disclination wall (‘grain boundary’). In a the two regions of opposite polar sense are of equal size; b shows the hypothetical E-field-induced shift in the distribution of conical conformers of **1** as a selective growth of the ‘up’ region (at the expense of the ‘down’) under the influence of an appropriately oriented poling field.

ing of mesogens and chromophores on the periphery of the cyclic macromolecule are anticipated¹¹ to promote statistical cone-like macromolecular shapes as schematically shown in Fig. 1b. Such conical shapes may pack to form a locally polar, suprastructure in which the cone's base-to-tip sense is propagated from layer to layer (Fig. 4a). However, both directions of such spontaneous local polarity—exaggerated in Fig. 4 as isolated regions of 'up' and 'down' packing separated by 'grain boundaries'—are equiprobable in the S_A phase. We therefore postulate that E-field poling promotes conformational transformations of **1** that shift the equilibrium distribution of its tertiary structures to favour one cone sense. This shift is depicted as a lateral growth of regions of the energetically favoured, polar sense of cone-packing at the expense of the opposite sense (Fig. 4b). The shifted distribution is 'pinned' and does not change after the removal of the E-field,

thereby accounting for the unusual persistence of the SHG above T_g and at the same time providing a plausible mechanism for its bistability.

Our findings emphasize that the delicate interplay between molecular design and sample fabrication can be important in stabilizing non-centrosymmetry in polymeric materials for NLO applications. This is particularly relevant for macromolecular materials as most polymer processing strategies are characterized by large (shear) stresses. The suggestion that polar (ferroelectric) packing might be exhibited by conical-shaped mesogens is not new¹⁹. But the E-field-induced structural transition (as determined from X-ray measurements) and the unusual temporal stability (and bistability) of the second-order NLO activity exhibited by **1** is the first experimental evidence for such a ferroelectric S_A phase^{20–22}. □

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1. Chémia, D. S. & Zyss, J. (eds) *Nonlinear Optical Properties of Organic Molecules and Crystals* (Academic, Orlando, 1987).
2. Schmidt, K. et al. *Liq. Cryst.* **14**, 1735–1752 (1993).
3. Singer, K. D., Sohn, J. E. & Lalama, S. J. *Appl. Phys. Lett.* **49**, 248–250 (1986).
4. Wu, J. et al. *Appl. Phys. Lett.* **58**, 225–227 (1991).
5. Marder, S. R. & Perry, J. W. *Science* **263**, 1706–1707 (1994).
6. Burland, D. M., Miller, R. D. & Walsh, C. A. *Chem. Rev.* **94**, 31 (1994).
7. Verbiest, T. et al. *Science* **268**, 1604–1608 (1995).
8. Walba, D. M. et al. *Mol. Cryst. Liq. Cryst.* **198**, 51–60 (1991).
9. Benne, I., Semmler, K. & Finkelmann, H. *Macromol. Rapid Commun.* **15**, 295–302 (1994).
10. Spes, P., Hessling, M. & Kreuzer, F.-H. US Patent No. 5231206 (1993).
11. Bunning, T. J., Klei, H. E., Samulski, E. T., Crane, R. L., Linville, R. J. *Liq. Cryst.* **10**, 445–456 (1991).
12. Bunning, T. J., Klei, H. E., Samulski, E. T., Adams, W. W. & Crane, R. L. *Mol. Cryst. Liq. Cryst.* **231**, 163 (1993).
13. Cull, B., Shi, Y., Kumar, S., Shih, R. & Mann, J. *Phys. Rev. E* **51**, 526–535 (1995).
14. Wang, H., Jarnagin, R. C. & Samulski, E. T. *Macromolecules* **27**, 4705–4713 (1994).
15. Marder, S. R. et al. *Science* **263**, 511–514 (1994).

16. Lindsay, G. A., Henry, R. A., Hoover, J. M., Knoesen, A. & Mortazavi, M. A. *Macromolecules* **25**, 4888–4894 (1992).
17. Man, H. T. & Yoon, H. N. *Adv. Mater.* **4**, 159–168 (1992).
18. Singer, K. D. & King, L. A. *J. Appl. Phys.* **70**, 3251–3255 (1991).
19. Xu, B. & Swager, T. M. *J. Am. Chem. Soc.* **115**, 1159–1160 (1993).
20. Petschek, R. G. & Wiefeling, K. M. *Phys. Rev. Lett.* **59**, 343–346 (1987).
21. Perchak, D. R. & Petschek, R. G. *Phys. Rev. A* **43**, 6756–6770 (1991).
22. Tournilhac, F. & Simon, J. *Ferroelectrics* **114**, 283–287 (1991).
23. Tam, W., Guerin, B., Calbrese, J. C. & Stevenso, S. H. *Chem. Phys. Lett.* **154**, 93–96 (1989).
24. Bierlein, J. D., Cheng, L. K., Wang, Y. & Tam, W. *Appl. Phys. Lett.* **56**, 423–425 (1990).
25. Bunning, T. J. thesis, Univ. Connecticut (1992).

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Direct mechanical measurement of interatomic potentials

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THE bonding potential between atoms determines all the key properties of matter. It is usually deduced from a wide variety of experimental parameters such as elastic moduli, binding energy and vibrational nonlinearities. These provide an indirect route to quantities such as virial coefficients which characterize the variation of potential energy with interatomic spacing¹. Here we report use of a modified atomic force microscope² to measure mechanically the interatomic forces between a tip and the sample surface as a function of separation. We use a magnetically controlled feedback mechanism to resist the 'jump to contact' that commonly occurs in mechanical force measurements at small separations, enabling us to map out reversible curves to separations closer than the point of inflection in the potential-energy curve. This method provides a direct means for continuous measurement of forces between atoms as they approach towards contact.

Directly mapping out interatomic forces, including strong interaction and even equilibrium separation, requires exploration of rapidly varying forces and gradients. Any real apparatus for force measurement also has a finite rigidity or spring constant. In the case of the atomic force microscope (AFM)² this is a lever on which the probing tip is mounted, the spring constant being fixed by the lever geometry and material. When the gradient of the tip-surface attractive interaction force, which is increasingly negative during the approach, exceeds the magnitude of the lever stiffness, the tip and surface will snap into contact, and then exhibit adhesion of the resulting solid-solid contact. To enable forces to be detected with reasonable sensitivity, a soft lever is normally used in AFM, but this in turn means that only large separations can be probed if the snap-in is to be avoided. Direct observation of forces between vacuum-separated surfaces has thus hitherto been restricted to long-range Van der Waals forces^{3,4} or to systems without feedback control of separation⁵.

We have now overcome the problems at smaller separations and larger force gradients by means of a force-controlled feedback system, which effectively increases the lever stiffness as tip and surface start to interact strongly. The feedback system maintains the AFM tip position constant by the application of force directly to the tip. As the sample approaches the tip, the resulting attractive interaction forces on the tip are therefore compensated by an opposing force generated by the feedback loop. This differs from normal AFM practice, in that tip force, rather than displacement, is driven—with displacement drive alone, the lever stiffness remains fixed. The applied force is controlled by current through a coil situated near the tip, the resulting field acting on a small piece of magnetic material mounted directly behind the tip⁶.

A further refinement allows us to accurately and continuously map the interaction force gradient whilst the tip-sample separation is varied. Superimposed on the feedback control force is a

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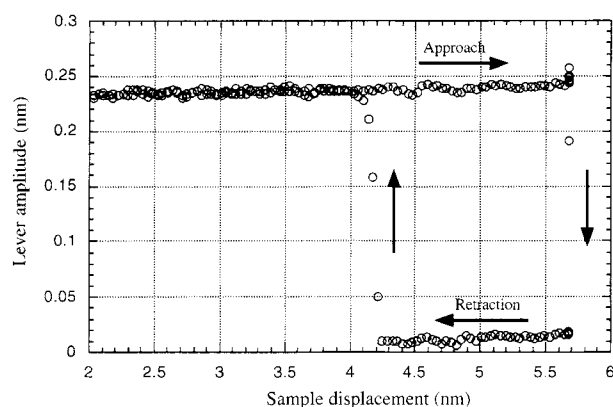


FIG. 1 Behaviour of the AFM without feedback control. During approach, the tip jumps in to the surface and there is a rapid fall in measured lever oscillation amplitude. On retracting the sample, the tip remains in contact with minimal change in contact stiffness until the point at which the lever jumps off the surface. Note that the actual tip jump-in distance is ~ 4.5 nm (not shown), as it is free to move without feedback control.

small-amplitude force modulation, at a frequency well above the unity gain frequency of the feedback loop, but well below the free-lever first resonance. The resulting modulated displacement of the tip is detected using lock-in techniques, so the force gradient between tip and surface can be directly determined by simply dividing the modulating force by the resulting displacement⁶. This a.c. lock-in method also gives much improved signal-to-noise performance compared to the raw force feedback signal. This noise advantage has been exploited elsewhere, in detecting molecular structuring of confined liquid in AFM⁷. We note that the stiffness determined at the modulation frequency is that of the lever unaffected by the feedback loop. For the apparatus used here the d.c. gain of the open loop is 150, and it has a single, dominant pole at 1 Hz. Unity gain is thus at 150 Hz. The modulation frequency used is 2.3 kHz, so the feedback loop will only reduce the modulated amplitude by 6%. As the lever first resonance is at 10.3 kHz, and has fairly high Q , we conclude that measurements at 2.3 kHz give the d.c. open-loop lever stiffness while retaining the stiffness amplifying effect of the feedback loop at lower frequencies. (The process is in this respect not unlike obtaining tunnelling barriers in STM by tunnel gap modulation at higher frequencies.) The rate of tip-sample approach is $7 \text{ \AA s}^{-1}$ with a step size of $0.35 \text{ \AA}$, designed to be significantly slower than the response time of the feedback circuit.

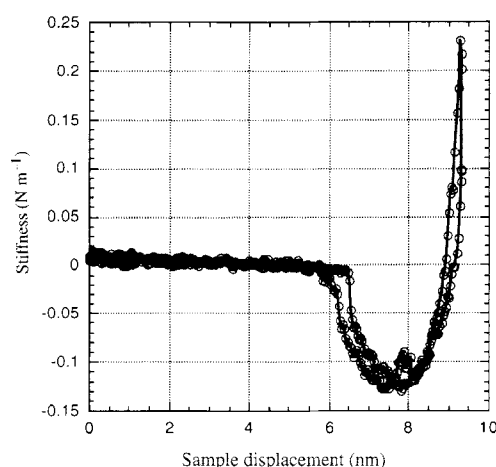
We use a modified Omicron ultrahigh vacuum (UHV) AFM, which is part of a comprehensive UHV system with a variety of surface analytical techniques; further details are given elsewhere⁸. The tip is silicon, on a silicon cantilever, and the sample is Si(111) prepared by standard procedures in the same apparatus. The free lever stiffness is 0.24 N m^{-1} (for the data reported here), which is subtracted from the overall stiffness measured by modulation to give the net tip-sample interaction stiffness. Note that the

effective d.c. stiffness of the lever with the feedback loop on is 37 N m^{-1} .

We first describe the behaviour without the force feedback. Figure 1 shows the lever oscillation amplitude resulting from the constant amplitude of force modulation, as the sample is first brought towards the tip, and then retracted. A slight rise in amplitude (negative interaction stiffness) occurs on approach as expected, in response to long-range interactions. Then at a critical separation snap-in occurs, and the oscillation amplitude suddenly falls to a very small value. As the modulating force is fixed, this means that the spring constant of the tip and surface interaction has suddenly risen to a very large positive value, over 10 N m^{-1} , implying a significant area of contact. The sample approach ramp is stopped as soon as this change in contact stiffness is observed. When the sample is then retracted, the contact stiffness remains large, until a sudden pull-off occurs, at a tensile force of several nanonewtons. The whole cycle involves considerable hysteresis, possibly some plasticity, and closely resembles both experimental observations of adhesion in UHV^{9,10}, and molecular-dynamics simulations^{11,12}.

The introduction of the force feedback loop brings a dramatic change in behaviour. Figure 2 shows a complete approach and separation cycle when the force feedback loop is active. Only moderate variations of lever modulation amplitude occur, and we plot the actual interaction stiffness variation, that is, overall stiffness minus lever stiffness. Some features are immediately apparent. First, and in striking contrast to Fig. 1, the behaviour is quite reversible. There is essentially no hysteresis between approach and retraction, even though we are exploring interactions very much stronger than those which cause snap-in when there is no feedback. (What little hysteresis is visible is almost certainly due to thermal drift and creep of the piezo material

FIG. 2 Behaviour of the AFM with feedback control. The lever position is held at a fixed point by applying a force to the tip. Measured stiffness of interaction during approach and retraction of the sample is now smooth and reversible, in contrast to Fig. 1.



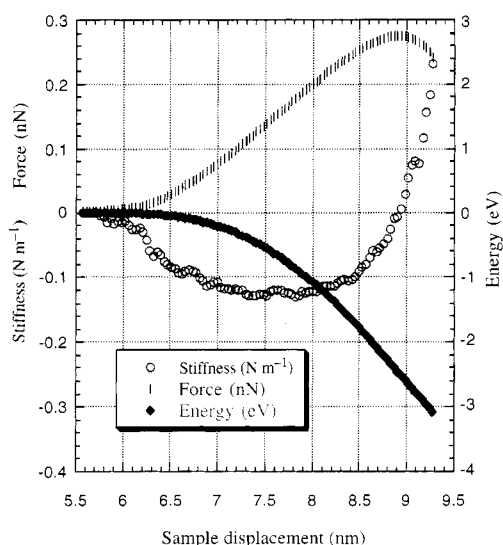


FIG. 3 Force, potential-energy and stiffness curves. The first two curves were calculated from the stiffness measurement by integration. The force is positive, tensile and reaches a maximum of just under 0.3 nN.

which controls the sample motion.) We are therefore observing a conservative potential interaction, and the whole cycle is smooth, reversible, controlled and stable. Second, the interaction stiffness starts out negative, as might be expected for an increasingly attractive force, but on closer approach, smoothly moves to become positive. In other words, the general shape strongly resembles that expected from a typical interatomic interaction potential—initial attraction at longer range and the onset of repulsion at shorter ranges. As the stiffness becomes positive (the tensile force passes its maximum) our measurement is reaching inside the point of inflection of the potential-energy curve. Simple integration gives the force, and potential energy, as a function of sample distance (Fig. 3). We note that the force can also be directly measured from the feedback signal, and indeed has the form and magnitude shown in Fig. 3, but the modulation-derived data has much improved signal to noise ratio, as expected for lock-in methods. The curves are repeatable, to within a few percentage points, between different approach cycles at the same point on the surface—neither tip nor surface are damaged by the interaction. The curve changes at different locations on the surface, yet retains the same general form. That is, the interaction measurement is sensitive to site.

How may these results be understood? It is at first tempting to suppose that a single interatomic pair potential is being mapped. However, it is clear that even if there were to be only two atoms interacting, we would not see just a single bond potential. At the very least, the back bonds will also stretch. The total measured displacement will be the sum of contributions due to a number of bonds in series and parallel and be significantly larger than a single

bond. This is evident from the displacement scale in Figs 2 and 3. We conclude that we are mapping the potential of a small group of atomic bonds—a ‘many-atom’ potential. Breaking down such a potential into individual atom pair potentials cannot generally be done by simple additive means, as shown in *ab initio* studies¹³. The local environment and coordination of the atoms is vital in determining bond interactions, and this is what we are in fact measuring. Figure 3 shows the energy curve point of inflection is at 2.5 eV, and that the maximum (tensile) force is just under 0.3 nN, not impossibly far from the expected ~ 1.5 nN for a single Si–Si bond, and rather larger than expected for a purely Van der Waals bond¹⁴. There is also a possibility that electrostatic forces may be present and contribute to the extended range.

A picture therefore emerges of the interaction occurring between the surface and a small cluster of atoms protruding from the end of the tip. This is hardly unexpected, and indeed the existence of such protruding atoms is generally thought to be the reason why scanning tunnelling microscopy (STM) functions at all. It is known that STM tip atoms can experience quite strong forces¹⁵ while remaining stable, and that tip forces can be used to manipulate the position of surface atoms¹⁶. However, despite its importance, the exact structure of the tip in STM remains unknown. Our data provides another possible avenue to characterize the tips.

It might be thought that once one part of the tip is in strong interaction with the surface, there should be a very rapid increase in contact area as described by continuum models of adhesion¹⁷. However, Greenwood has recently shown¹⁸ that provided the parameter $\mu = (R/\epsilon^2/E^2\epsilon^3)^{1/3}$ is less than 1, then the whole continuum system does not in fact exhibit any elastic instabilities, provided the apparatus is sufficiently rigid. A low value of the Tabor parameter μ (ref. 19) indicates that a significant fraction of the surface forces are acting outside the zone of intimate contact. Our estimated values of tip radius $R < 20$ nm (typical for these tips, from SEM), surface energy γ (~ 0.5 J m⁻²), reduced elastic moduli E^* ($\sim 10^{11}$ N m⁻²), and surface force range ϵ (~ 3 Å) are such that $\mu \ll 1$ and so there should be no instability, provided the lever is sufficiently stiff.

Our data also indicate that at larger separations there is a more slowly varying Van der Waals background force, due to the larger-radius, mesoscopic tip. However, on close approach the projecting few atom tips become more important. If there is no feedback, these few atom tips can control the snap-in, which thus might occur rather sooner than the longer-range, mesoscopic tip force gradient alone would indicate. On snap-in, the few atom tips could be deformed owing to the impact, giving larger adhesion. Such tip crashes are suppressed by our new feedback method.

Our results have some further consequences. It is clear that this stable feedback control of separation in the presence of relatively strong bond interactions can be used during raster scanning in AFM, and therefore that atomic resolution should be possible²⁰. But unlike existing atomic-resolution AFM, which uses very large-amplitude tip oscillations^{21–23}, our new method means that low modulation amplitudes can be used, so that force–distance spectroscopy of specific sites is possible—mechanical characterization of the potentials of specific chemical bonds. □

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- Pettifor, D. G. *Bonding and Structure of Molecules and Solids* (Clarendon, Oxford, 1995).
- Binnig, G., Quate, C. F. & Gerber, Ch. *Phys. Rev. Lett.* **56**, 930–933 (1986).
- Tabor, D. & Winterton, R. H. S. *Nature* **219**, 1120–1121 (1968).
- Israelachvili, J. N. *Intermolecular and Surface Forces with Applications to Colloidal and Biological Systems* (Academic, London, 1985).
- Dürig, U., Züger, O. & Pohl, D. W. *Phys. Rev. Lett.* **65**, 349–352 (1990).
- Jarvis, S. P., Oral, A., Weins, T. P. & Pethica, J. B. *Rev. Sci. Instrum.* **64**, 3515–3520 (1993).
- O’Shea, S. J., Welland, M. E. & Pethica, J. B. *Chem. Phys. Lett.* **223**, 336–340 (1994).
- Jarvis, S. P., Yamada, H., Yamamoto, S.-I. & Tokumoto, H. *Rev. Sci. Instrum.* **67**, 2281–2285 (1996).
- Pashley, M. D., Pethica, J. B. & Tabor, D. *Wear* **100**, 7–31 (1984).
- Howald, L., Luethi, R., Meyer, E., Guethner, P. & Guentherodt, H.-J. *Z. Phys. B* **93**, 267–268 (1994).
- Sutton, A. P. & Pethica, J. B. *J. Phys. C* **2**, 5317–5326 (1990).
- Landmann, U., Luedtke, W. D., Burnham, N. A. & Colton, R. J. *Science* **248**, 454–461 (1990).
- Heine, V., Robertson, I. J. & Payne, M. C. *Phil. Trans. R. Soc. A* **334**, 393–405 (1991).
- Sutton, A. P. *Electronic Structure of Materials* (Oxford Univ. Press, 1993).
- Clarke, A. R. H. et al. *Phys. Rev. Lett.* **76**, 1276–1279 (1996).
- Eigler, D. M. & Schweizer, E. K. *Nature* **344**, 524–526 (1990).
- Johnson, K. L., Kendall, K. & Roberts, A. D. *Proc. R. Soc. Lond. A* **324**, 301–313 (1971).
- Greenwood, J. A. *Proc. R. Soc. Lond. A* (submitted).
- Tabor, D. J. *Colloid Interface Sci.* **58**, 2–13 (1977).
- Perez, R., Payne, M. C. & Simpson, A. D. *Phys. Rev. Lett.* **75**, 4748–4751 (1995).
- Giesibl, F. J. *Science* **267**, 68–71 (1995).
- Kitamura, S. & Iwatsuki, M. *Jpn. J. Appl. Phys.* **34**, L145–L148 (1995).
- Sugawara, Y., Ohta, M., Ueyama, H. & Morita, S. *Science* **270**, 1646–1648 (1995).

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Self-assembly of organic films on a liquid metal

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The structure and phase behaviour of organic thin films result from the subtle interplay of intermolecular Van der Waals interactions, which promote self-assembly and long-ranged order, and the more complex interactions between the end groups of the organic chains and the substrate. The structure of molecular films of amphiphiles has been extensively studied on subphases of dielectric liquids, notably water (Langmuir monolayers) and on solid surfaces (self-assembled monolayers, SAMs)^{1–4}. Here we report structural studies, by synchrotron X-ray scattering, of an intermediate case: densely packed alkanethiol films on the surface of liquid mercury. While, like SAMs, these films form strong chemical bonds to the subphase, this subphase is smooth and unstructured, as in the case of Langmuir monolayers. But unlike either of these^{1,2,5–7}, our films have no in-plane long-range order. We suggest that the strong interaction of the thiol group with the underlying disordered liquid dominates here over the order-promoting interactions of the alkyl chains.

The significantly different structures of SAMs and Langmuir monolayers composed of similar chain-like molecules reflect fundamental differences in the subphase–end-group interactions. First, the bond strengths of SAMs to solid substrates (typically a few hundred kilojoules per mole) greatly exceed those of the typical hydrogen bonds of Langmuir monolayers to water or organic subphases ($\leq 10 \text{ kJ mol}^{-1}$)⁸. Second, the liquid subphase molecules can rearrange freely and lack intrinsic in-plane order, whereas the crystalline SAM substrates possess well defined long-range order. Accordingly, the order in alkanethiols on crystalline gold, the best-studied SAM³, is induced epitaxially by the strong (418 kJ mol^{-1})⁹ Au–S bond. Even the corrugation potential, the 5–20% spatial modulation of the binding energy induced by the substrate's periodic structure, still exceeds considerably the inter-chain interactions of the alkylthiols ($\leq 1 \text{ kJ mol}^{-1}$ per CH_2 unit)⁸. In Langmuir monolayers, by contrast, the order is induced by inter-chain interactions, which exceed the hydrogen-bond strength in molecules longer than 10–15 carbons.

Alkanethiols on liquid mercury provide a system which can, in principle, help to disentangle the influence of adsorbate–substrate bond strength from the lattice periodicity. The liquid mercury surface lacks long-range order, while the Hg–S binding energy ($\sim 200 \text{ kJ mol}^{-1}$)⁹ is comparable to those of Au–S, Ag–S and Cu–S ($200\text{--}400 \text{ kJ mol}^{-1}$)⁹ and is over ten times stronger than the hydrogen bonds of Langmuir monolayers. Unlike their solid counterparts, the ultra-smooth liquid metal subphases are free from any static structural surface features like corrugations, atomic steps and defects. Much thermodynamic and kinetic data exists for organic films on mercury (both at the vapour¹⁰ and electrochemical interfaces^{11,12}), but no direct structural information has previously been available.

We have investigated alkanethiols, $\text{CH}_3(\text{CH}_2)_n\text{SH}$ (denoted C_n) with $n = 8, 12, 16, 18, 22$ and 30 on clean liquid mercury surfaces by grazing-incidence X-ray diffraction (GIXD), a technique sensitive to the molecular structure within the surface plane, and X-ray reflectivity, which probes the surface-normal electronic density profile. Both techniques have been applied recently to studies of the clean surfaces of liquid mercury^{13,14} and liquid gallium¹⁵, revealing a liquid-like in-plane arrangement of the surface atoms¹⁴, atomic stratification in the surface normal direction and sub-ångström roughness^{13,15}. $R(q_z)$, the measured reflectivity profile for wavevector q_z of the liquid mercury surface covered by C_{18} is shown in Fig. 1 (circles). The data closely follow the reflectivity R_{Hg} of clean liquid mercury at low q_z and both are only slightly lower than the Fresnel reflectivity R_f of an ideally flat surface. This indicates a similar, small surface roughness for the bare and thiol-covered mercury surfaces. At $q_z > 0.5 \text{ \AA}^{-1}$, R dips significantly below R_{Hg} but approaches it again at the broad peak around $2.16 \text{ \AA}^{-1}$. As this peak characterizes the atomic surface layering of the mercury subphase¹³, its persistence for the thiol-covered surface indicates that the organic film induces no major changes in the liquid-metal surface structure. The most significant change, compared with the bare mercury surface, is the emergence of periodic oscillations.

The Fresnel-normalized reflectivity (Fig. 2) further highlights these features. The period of the five oscillations observed in Fig. 2a, $\Delta q_z \approx 0.25 \text{ \AA}^{-1}$, corresponds to a layer thickness $d = 2\pi/\Delta q_z = 25 \text{ \AA}$, in agreement with the length of a fully extended C_{18} molecule. Similar features are observed for thiols of different lengths n . For example, R/R_f of the C_{12} monolayer (Fig. 2b) has a very similar overall shape to Fig. 2a but the oscillation

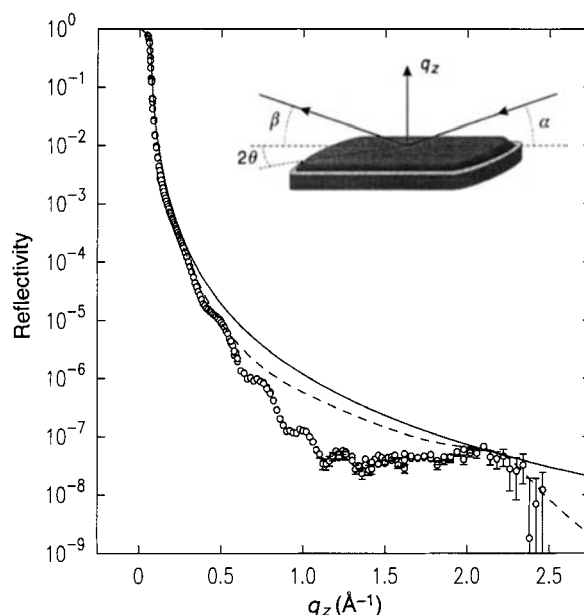


FIG. 1 X-ray reflectivity from a C_{18} ($\text{CH}_3(\text{CH}_2)_{17}\text{SH}$) monolayer-covered (circles) and a bare (dashed line) liquid mercury surface. The Fresnel reflectivity R_f (solid line) is also shown. The presence of the monolayer is clearly indicated by the oscillatory modulations of the reflectivity. The inset shows the experimental geometry. Alkanethiols were deposited by direct application of the thiols in their liquid state, by chemical vapour deposition, by self-assembly from an ethanol bath, or by spreading from a dilute chloroform solution. All techniques yielded well defined monolayers. X-ray reflectivity measurements (at wavelengths $0.65 \text{ \AA} \leq \lambda \leq 1.2 \text{ \AA}$) were carried out at beamline X22B¹³ and wiggler beamline X25¹⁵ at the National Synchrotron Light Source (Brookhaven) at room temperature and under nitrogen. Exposure to the beam was minimized to avoid beam damage. The absolute reflectivity was obtained from measurements along the specular axis ($2\theta = 0^\circ$), subtracted by the diffuse background (measured at $2\theta = \pm 0.6^\circ$) and normalized to the direct beam intensity.

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period is increased to $\Delta q_z = 0.36 \text{ \AA}^{-1}$, corresponding to the shorter length of this molecule $d = 2\pi/\Delta q_z = 17.5 \text{ \AA}$. Oscillations which scale with the length of the C_n molecule have been observed for all thiols studied ($8 \leq n \leq 30$), demonstrating a very similar monolayer structure for all lengths. For long chains ($n = 22, 30$), however, the oscillations are weak and the reflectivity data indicates a high interfacial roughness.

Quantitative information on the atomic arrangement along the surface-normal direction is obtained by fitting the reflectivity data to a density-dependent model reflectivity calculated from the Born approximation¹⁶. The fitted profiles, displayed as solid lines in Fig. 2 with identical parameters for all data sets (apart from the number of CH_2 groups), are in excellent agreement with the experimental data. The corresponding density profiles are shown in Fig. 3. Comparison with the density profile for the clean mercury surface reveals the extended hydrocarbon tail, and the higher-density sulphur atom between the mercury and the hydrocarbon tail. The slight decrease observed in the amplitudes of the first mercury layers probably reflects the small increase in surface roughness expected by the capillary-wave model from a decrease in the surface tension due to the monolayer's presence. Despite this, the general features of a layered mercury surface with an adsorbed, densely packed monolayer of fully extended molecules are found in all the systems studied. Also, the surface roughness (estimated from the density profile width of the first mercury layer) remains about $1 \text{ \AA}$, that is, in good agreement with the value given by capillary-wave theory¹³.

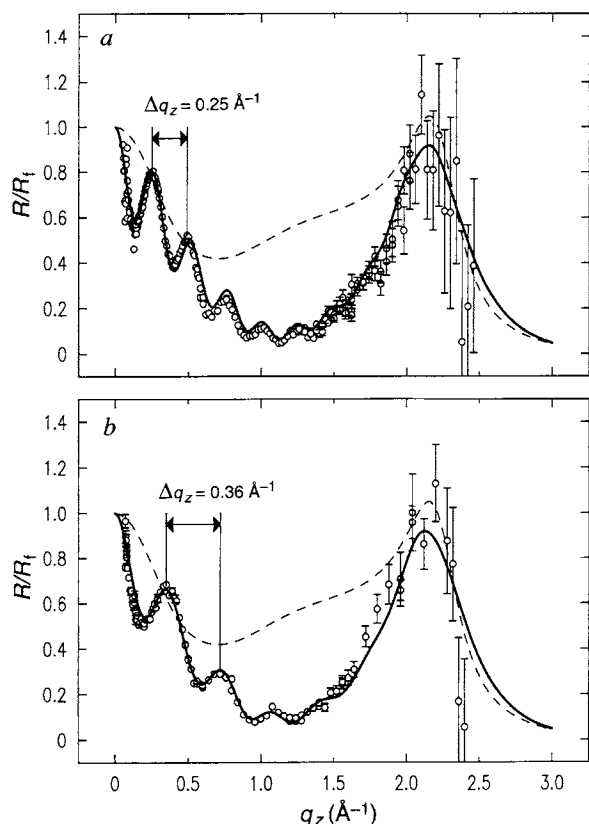


FIG. 2 The measured normalized reflectivity R/R_i of C_{18} (a) and C_{12} (b) monolayers on liquid mercury (circles) and of the bare mercury surface¹³ (dashed line). The different periodicities Δq_z for C_{18} and C_{12} are indicated. Solid lines are fits to a model used to describe the atomic layering at the bare mercury surface¹³, modified by adding $n + 1$ gaussians representing the n carbon units and the terminal sulphur, respectively. The area per molecule of the thiols was fixed at that of the high-coverage multilayer phase ($19.23 \text{ \AA}^2$ according to the GIXD diffraction pattern). The fits in a and b differ only in the number of CH_2 groups.

Because of the high adlayer density ($0.34 \pm 0.02 \text{ e \AA}^{-3}$), which is close to that found in crystalline alkanes and significantly higher than in the melt, in-plane ordering of the molecules might be expected. Despite an exhaustive search at two of the most intense beamlines worldwide, no sharp in-plane peaks corresponding to an ordered adlayer structure were found for the monolayer phases of thiol molecules of any of the examined lengths. In particular, a careful search was done from 1.5 to $1.7 \text{ \AA}^{-1}$ where structurally similar SAMs and Langmuir monolayers show the lowest-order in-plane peaks. The GIXD data is shown in Fig. 4 for the clean and C_{18} -covered mercury surface. Both show the characteristic broad peaks around the in-plane wavevectors $q_{\parallel} \approx 2.3 \text{ \AA}^{-1}$ and $q_{\parallel} \approx 4.5 \text{ \AA}^{-1}$ expected for liquid mercury¹⁴ although at different relative intensities, which may indicate a thiol-induced modification of the mercury surface structure. As we discuss below, the absence of in-plane order in the densely packed alkanethiol monolayer is probably promoted by the disordered mercury subphase via the strong, covalent Hg-S bond. We note that at thiol coverages greater than a monolayer, sharp, resolution-limited in-plane peaks are readily observed, corresponding to the formation of well-ordered multilayer phases (O.M.M. *et al.*, manuscript in preparation).

The results presented here highlight the importance of the relative strengths of the substrate-end-group and inter-chain interactions and the specific order preferred by each. In systems where the latter dominate, such as densely packed films of alkanes, alcohols and fatty acids on pure water^{1,2,17}, long-range,

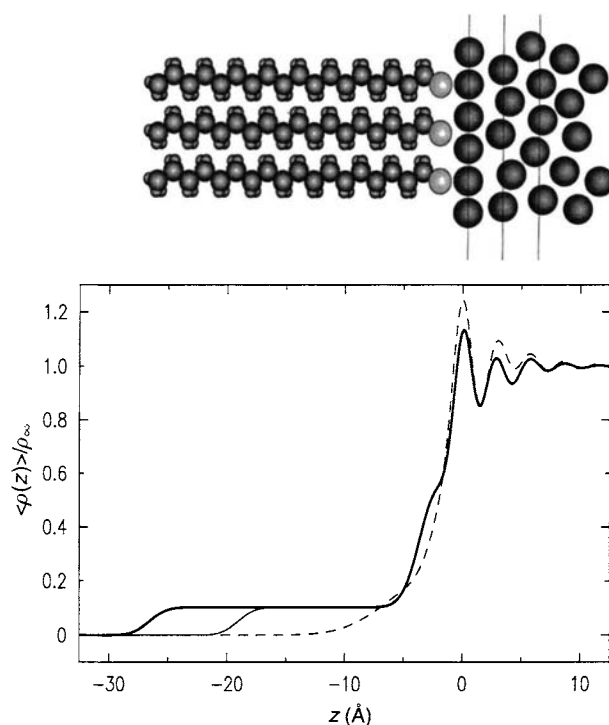


FIG. 3 Schematic real-space model and normalized electron density profiles $\langle \rho(z) \rangle / \rho_{\infty}$ (where ρ_{∞} is the bulk electron density of mercury) obtained from the fits for C_{18} (bold line) and C_{12} (thin line). The upper and lower figures are aligned with each other. Vertical lines in the model illustrate the position of the three outermost surface layers of mercury, with the origin of z coinciding with the first mercury layer. The carbon-carbon and the carbon-sulphur spacing along the surface normal were fixed at 1.25 and $1.5 \text{ \AA}$, respectively. These spacings correspond to the projections of the carbon-carbon ($1.53 \text{ \AA}$) and the carbon-sulphur ($1.82 \text{ \AA}$) bonds onto the molecular axis. As the gaussian width describing the CH_2 groups (typically $1.2 \text{ \AA}$) is considerably greater than half the separation between the carbon atoms, the density profile appears constant in the central part of the chain.

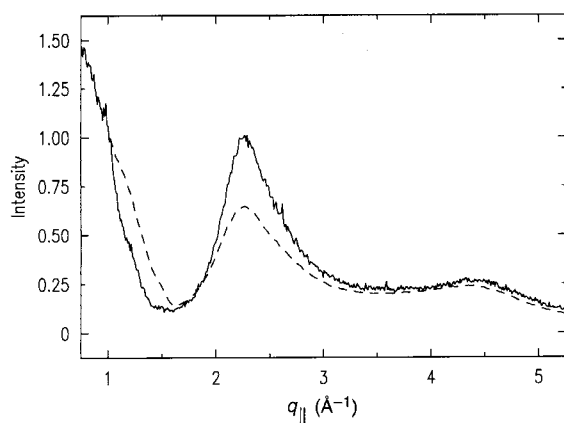


FIG. 4 Grazing-incidence diffraction patterns of a bare (dashed line) and a C_{18} monolayer-covered (bold solid line) mercury surface, measured with an incidence angle $\alpha = 0.2^\circ = 0.6\alpha_c$ (where α_c is the critical angle) and a vertical detector acceptance of $0 \leq q_z \leq 1.1 \text{ \AA}^{-1}$ (intensity given in arbitrary units). Here $q_{\parallel} = 4\pi/\lambda \sin \theta$ is the in-plane momentum transfer and θ is defined in Fig. 1 inset. Only the broad peaks corresponding to the mercury liquid structure factor are found in the monolayer data (in-plane resolution $\Delta(2\theta) = 5 \text{ mrad}$). This data was obtained at the TROIKA undulator beamline of the ESRF¹⁹. GIXD measurements at X22B and X25 at NSLS gave similar results.

in-plane order is usually found. This order results not only from the confinement of the monolayer to the water surface, because such in-plane order also appears in alkanes where crystalline monolayers form at the surface of their own melts at temperatures up to a few degrees above the bulk freezing point¹⁸. In this case the 'monolayer' and 'subphase' molecules are identical, and, of course, fully miscible. Both systems are dominated by the inter-chain interactions and the bonds to the sub-phase ($\sim 10 \text{ kJ mol}^{-1}$ hydrogen bonds in Langmuir monolayers and $\sim 1 \text{ kJ mol}^{-1}$ van der Waals interactions in alkanes) are relatively weak. Thus, we conclude that the inter-chain interactions are responsible for the long-range order in these systems. The interactions with the disordered atoms of the liquid subphase tend, in principle, to disrupt this ordering but are too weak to cause more than slight changes in the structure and the phase diagrams of these films.

For SAMs, spatial variations of the interactions with the substrate (corrugation potential) may either promote or oppose the ordering favoured by the inter-chain interactions, depending on the spatial periodicities of both interactions. For alkanethiols on Au(111) (refs 5–7) this corrugation potential favours well ordered monolayers which may be either commensurate or uniaxial incommensurate with the underlying metal. The small mismatch between the underlying substrate and alkane spacings is compensated by a molecular tilt. The elevated melting temperature of C_n on Au(111) relative to that of the bulk⁵ manifests the strong ordering tendency imposed by the interactions with the substrate.

Thiols on mercury 'feel' a similarly strong subphase interaction ($\sim 200 \text{ kJ mol}^{-1}$), but as the liquid mercury surface has no intrinsic long-range order, there is no underlying corrugation potential to order the thiol monolayer. This should promote formation of the order favoured by the inter-chain interaction. If, on the other hand, the ordered thiol layer were to impose its order on the underlying mercury through the strong subphase interaction, with the same structure as found for alkanethiols formed on gold and silver, then the resultant 5–10% compression of the mercury atoms would carry a great energy cost. In addition, this ordered mercury layer, at a temperature where the bulk is disordered, would represent a large increase in the entropic contribution to the free energy of the mercury and for these two reasons seems unlikely. Instead the (liquid-like) subphase surface structure is imposed on the adsorbate film, resulting in an in-plane-disordered film. □

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- Knobler, C. M. *J. Phys.* **3**, 17–22 (1991).
- Als-Nielsen, J., Jacquemain, D., Kjaer, D., Leveiller, F. & Leiserowitz, L. *Phys. Rep.* **246**, 252–313 (1994).
- Ulman, A. *An Introduction to Ultrathin Organic Films: From Langmuir Blodgett to Self Assembly* (Academic, Boston, 1991).
- Tredgold, R. H. *Order in Thin Organic Films* (Cambridge Univ. Press, 1994).
- Fenter, P., Eisenberger, P. & Liang, K. S. *Phys. Rev. Lett.* **70**, 2447–2450 (1993).
- Strong, L. & Whitesides, G. M. *Langmuir* **4**, 546–558 (1988).
- Chidsey, C. E. D., Liu, G.-Y., Rowentree, P. & Scoles, G. J. *J. Chem. Phys.* **91**, 4421–4423 (1989).
- Rappe, A. K. et al. *J. Am. Chem. Soc.* **114**, 10024–10035 (1992).
- Weast, R. C., Astle, M. J. & Beyer, W. H. (eds) *CRC Handbook of Chemistry and Physics* 66th Edn F174–F184 (CRC Press, Boca Raton, 1986).
- Smith, T. *Adv. Colloid Interface Sci.* **3**, 161–221 (1972).
- De Levie, R. *Chem. Rev.* **88**, 599–609 (1988).
- Buess-Herman, C. *Prog. Surf. Sci.* **46**, 335–375 (1994).
- Magnussen, O. M. et al. *Phys. Rev. Lett.* **74**, 4444–4447 (1995).
- Barton, S. W. et al. *Nature* **321**, 685–687 (1986).
- Regen, M. J. et al. *Phys. Rev. Lett.* **75**, 2498–2501 (1995).
- Pershan, P. S. & Als-Nielsen, J. *Phys. Rev. Lett.* **52**, 759–762 (1984).
- Weinbach, S. P. et al. *Adv. Mat.* **7**, 857–862 (1995).
- Wu, X. Z. et al. *Phys. Rev. Lett.* **70**, 958–961 (1993).
- Grübel, G., Als-Nielsen, J. & Freund, A. K. *J. de Phys. IV* **4**, 27–34 (1994).

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Prediction of global rainfall probabilities using phases of the Southern Oscillation Index

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THE El Niño/Southern Oscillation (ENSO) is a quasi-periodic interannual variation in global atmospheric and oceanic circulation patterns, known to be correlated with variations in the global pattern of rainfall^{1–3}. Good predictive models for ENSO, if they existed, would allow accurate prediction of global rainfall variations, thus leading to better management of world agricultural production^{4,5}, as well as improving profits and reducing risks for farmers^{6,7}. But our current ability to predict ENSO variation is limited. Here we describe a probabilistic rainfall 'forecasting' system that does not require ENSO predictive ability, but is instead based on the identification of lag-relationships between values of the Southern Oscillation Index, which provides a quantitative measure of the phase of the ENSO cycle, and future rainfall. The system provides rainfall probability distributions three to six months in advance for regions worldwide, and is simple enough to be incorporated into management systems now.

Climate forecast systems offer a means to reduce risks in potentially 'bad' years and maximize profits in potentially 'good' years. The Southern Oscillation Index (SOI) provides a measure that can be used in developing a seasonal forecast system. The SOI is here defined as the difference in atmospheric pressure anomalies between Tahiti and Darwin divided by the standard deviation of the difference (and multiplied by 10)⁸. The SOI is moderately correlated with future seasonal rainfall in some regions^{9,10}, partly due to the strong auto-correlation between SOI values in early austral winter with those three to six months ahead¹¹. This 'phase-locking' of the SOI into the annual cycle permits seasonal forecasting to be attempted once the early stages of various manifestations of the ENSO cycle are underway. In other words,

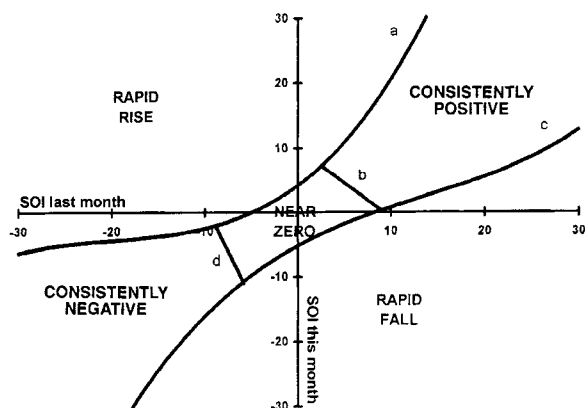


FIG. 1 Categorization of the five SOI phases, depending on SOI values in the current and immediately preceding month. The five SOI phases are: 'consistently negative', 'consistently positive', 'rapid fall', 'rapid rise' and 'consistently near zero'. For these regions, the current and previous month means, averaged over each region, are respectively $(-12.18, -14.52)$, $(+9.49, +11.21)$, $(-9.92, +2.75)$, $(+6.62, -4.38)$ and $(-1.67, -0.36)$ ¹². The boundaries between the regions (phases) in the figure are defined as follows: curve a, $M_2 = 4.2 + 1.07M_1 + 0.048M_1^2 + 0.0008M_1^3$; curve b, $M_2 = 10 - 1.08M_1$; curve c, $M_2 = -5.2 + 0.78M_1 - 0.024M_1^2 + 0.0006M_1^3$; curve d, $M_2 = -27.8 - 2.9M_1$. Here M_1 is the SOI value for the first month, and M_2 is the value for the second month¹⁹. The latest monthly SOI and the current SOI phase are available on <http://www.dpi.qld.gov.au/longpdk>.

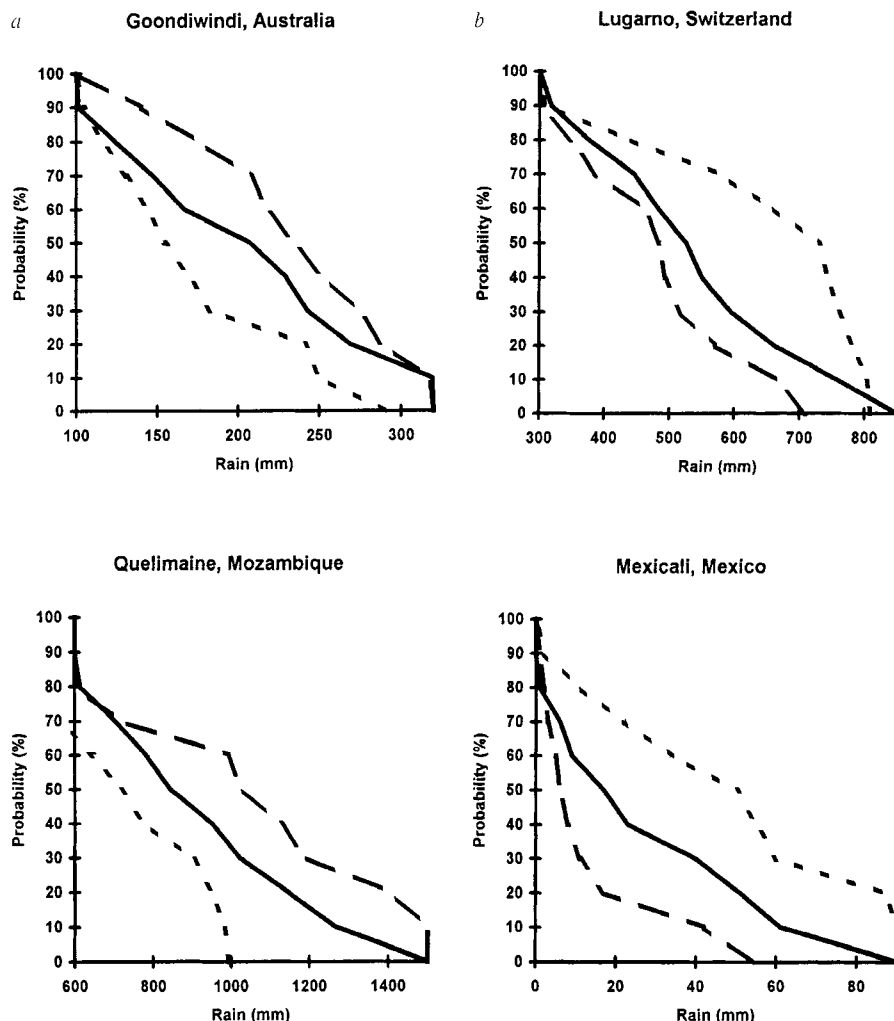
a forecast system based on 'phase-locking' of the SOI does not depend on a precise forecast of an El Niño event. Here we categorize current SOI patterns and relate these patterns to future rainfall probabilities based on lag-relationships derived from historical SOI patterns and rainfall over the past 120 years.

Determining the most appropriate SOI pattern to relate to rainfall is made possible through classification (using principal-components analysis and cluster analysis) of SOI behaviour¹². Every month of the past 120 years has been categorized into one of five SOI types or 'phases' that takes into account both the value and change in SOI. Earlier work demonstrated surprisingly little contamination or noise in monthly SOI data due to the intra-

seasonal oscillation¹³ in monthly SOI data. This point is reinforced by the fact that there is little difference in the effect of the intra-seasonal oscillation on correlations between the SOI and tropospheric variables if either monthly or seasonal data are used¹⁴. Further, the approach of using monthly SOI data has the advantage that the structure of the troposphere in relation to the Southern Oscillation is better identified. The coordinates of the boundaries of the five phases (together with a detailed description of the SOI phases) are depicted in Fig. 1. Further explanation of the SOI phase method may be found in a recent application to frost 'forecasting'¹⁵ and in an estimation of peanut yields¹⁶.

For every month of the year, rainfall probability distributions

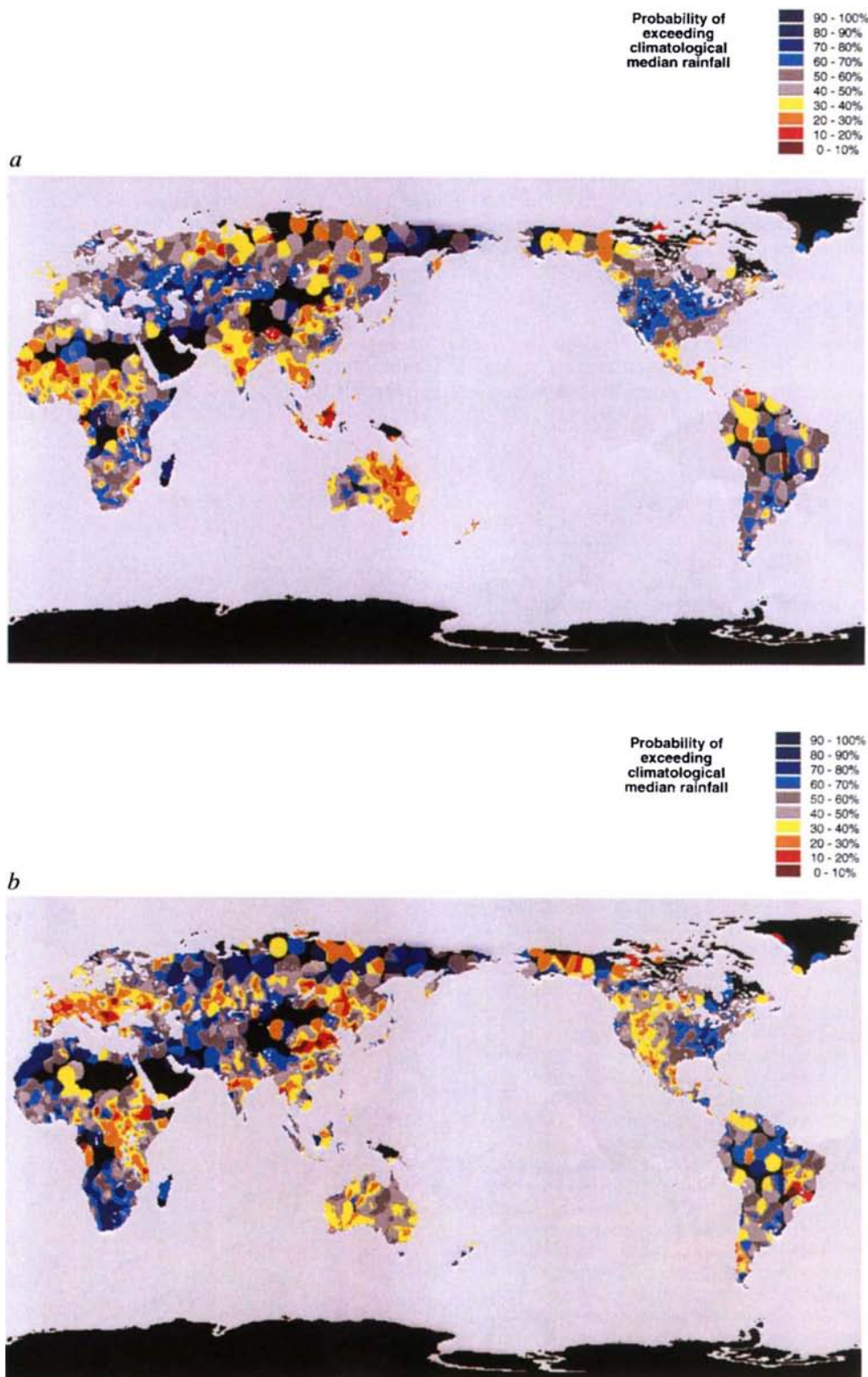
FIG. 2 Cumulative probability distributions of subsequent rainfall associated with SOI phases at the four locations mentioned in the text. The figures show probability of exceeding any particular rainfall amount. At most locations the data set includes at least the past 70 years, although this criterion could not be met in every instance. Broken lines represent years associated with each of two SOI phases; the solid line shows the 'all-years' distribution (climatology). At Goondiwindi, rainfall is for the period June–October. The short-dashed line depicts the cumulative distribution associated with SOI phase 'rapid fall' in the preceding April/May; the long-dashed line shows the distribution following SOI phase 'rapid rise'. The diagram shows that the probability of obtaining 200 mm at rainfall at Goondiwindi is 25% following SOI phase 'rapid fall' and 70% following SOI phase 'rapid rise', and that there is a difference in median rainfall between distributions of 80 mm. At Quelimaine, rainfall is for the period December–February. The short-dashed line shows the distribution associated with SOI phase 'consistently negative' in the preceding October/November, while the long-dashed line represents the distribution following SOI phase 'consistently positive'. The diagram shows that there is a 0% probability of rainfall exceeding 1,000 mm following SOI phase 'consistently negative', but a 65% probability of exceeding that amount following phase 'consistently positive'. At Lugarno, for rainfall in the May–July period, the short-dashed line shows higher rainfall as more likely following phase 'consistently negative' in March/April compared to phase 'rapid fall' (long-dashed line). At Mexicali, for rainfall in the October–December period, higher rainfall is more probable following phase 'consistently negative' (short-dashed line) in July/August than following phase 'consistently positive' (long-dashed line).



(for the subsequent three-month period), have been produced from the subsets of years corresponding to each of the five SOI phases; this has been done for locations with sufficient monthly rainfall record (>70 years)¹⁷. Figure 2 shows examples of such lagged cumulative rainfall probability distributions associated with two representative SOI phases for four globally dispersed locations: Goondiwindi, Australia; Quelimaine, Mozambique; Lugarno, Switzerland; and Mexicali, Mexico. At each of these locations there is a significant shift in a large part of the distribution associated with the SOI phases. Non-parametric statistical

'tests' (for example, Kruskal–Wallis and Kolmogorov–Smirnov) can be applied to determine whether the rainfall probability distributions (not just the rainfall medians) differ significantly. Applying these tests to the locations in Fig. 2 indicated that the cumulative probability distributions differed significantly at the 95–99% probability level. When such tests are applied at many locations there is, of course, a possibility that significant differences identified at some locations are there by chance; it is also the case that non-significant differences suggested at some other stations may, in fact, be real.

FIG. 3 *a*, Probability of exceeding climatological median rainfall for the August–October period associated with SOI phase 'consistently negative' in June/July. Based on individual analysis of 5,000 locations with >70 years of monthly data. Regions shaded dark brown to red represent the lowest probabilities of exceeding climatological median rainfall (0–20%), those shaded light orange to yellow represent probabilities between 20 and 40%, and so on. Black areas represent regions with insufficient data to perform this analysis. *b*, As *a* but depicting probability of exceeding climatological median rainfall for the September–November period following SOI phase 'rapid fall' in the preceding July/August.



Although the associations vary, there is generally a higher probability of receiving any given 'future' rainfall amount following the 'consistently positive' SOI phase than there is following the 'consistently negative' SOI phase at locations such as Goondiwindi and Qelimaïne. There is also, generally, a higher probability of receiving any given rainfall amount following SOI phase 'rapid rise' in the austral autumn (which may be associated with some breakdown of an El Niño event) than there is following SOI phase 'rapid fall'. Rainfall distributions following SOI phase 'consistently near zero' are usually (but not always) close to the 'all-years climatology' rainfall distribution. Although there is a significant shift in the cumulative probability distributions of subsequent rainfall associated with SOI phases at these locations, the rainfall range may be little altered.

Cumulative rainfall probability distributions for Lugarno and Mexicali demonstrate dissimilar associations with the SOI phases to those represented for locations such as Goondiwindi and Qelimaïne (Fig. 2). At Lugarno and Mexicali, the probability of obtaining any future given amount of rainfall for the May–July and October–December periods, respectively, is highest following the 'consistently negative' SOI phase. This type of relationship for Europe has been hinted at by others¹⁸.

A spatial representation of this analysis was developed by considering the probability of exceeding the climatological median for any season following each SOI phase. Figure 3a shows an example of a map depicting the probability of exceeding the climatological median rainfall for August–October associated with 'consistently negative' SOI phase in June/July. Figure 3b shows (as an example) the probability of exceeding the climatological median rainfall for September–November associated with the 'rapid fall' SOI phase in July/August. These are just two examples from the 60 maps produced using this method. The maps provide a 'snapshot' of the lag-relationships between SOI phase and rainfall. A simple least-squares contouring algorithm was applied to produce the spatial patterning following analysis of global rainfall data, although where data were scarce this resulted in an occasional circular pattern.

The maps show some remarkable features. In Fig. 3a, the 'consistently negative' SOI phase in June/July is associated with a 'low' probability of exceeding the climatological median rainfall for the subsequent three months in many world regions including much of eastern Australia, Indonesia, West Indies, Alaska, central India, Vietnam, northern parts of the former Soviet Union and sections of western Africa. Conversely, many world regions such as southwestern and north-central United States, Iran, central Asia and parts of South America have relatively high probabilities of exceeding their respective climatological median rainfalls. In Fig. 3b, the 'rapid fall' SOI phase is associated with a relatively low probability (0–40%) of exceeding climatological median rainfall for the subsequent three months over much of central Europe and central China. Conversely, much of South Africa, Namibia, north-west Africa, Iran and the eastern United States have a reasonably high probability (60–90%) of exceeding the climatological median rainfall for the following three months. Note that areas of 'no skill' (that is, close to 50% chance of exceeding the climatological median) are depicted as 'grey areas'. We were

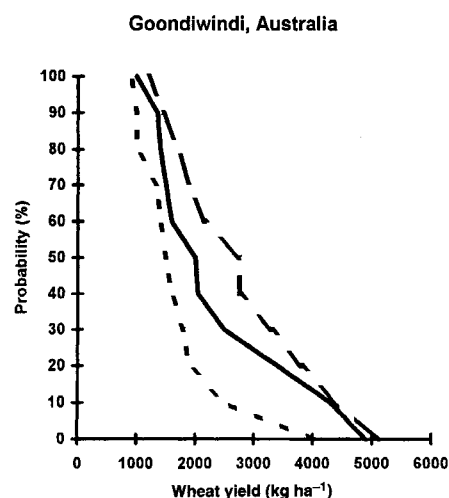


FIG. 4 Cumulative probability distribution of simulated wheat yield at Goondiwindi, Australia. The solid line represents the 'all-years' (100 years) distribution. The short-dashed line represents the probability of exceeding a given simulated yield following SOI phase 'rapid fall' in April/May, while the long-dashed line represents probability of exceeding a given simulated yield following SOI phase 'rapid rise'. The figure shows, for example, that the probability of exceeding 2,000 kg ha⁻¹ following SOI phase 'rapid fall' is 18%, while the probability of exceeding that amount following SOI phase 'rapid rise' is 64%.

surprised that a significant relationship between SOI phase and subsequent rainfall existed in northern parts of the former Soviet Union as this region had hitherto not been regarded as being strongly influenced by ENSO teleconnections (although maps in some references hint that this may be so¹⁸). Closer examination of our data revealed a significant difference at the 95% level in the cumulative distributions for rainfall in the July–September period, for example, associated with the SOI phases in May/June for locations in the former USSR such as Volochanka (lat. 70.95°, long. 94.50°) and Dudinka (lat. 69.40°, long. 86.17°).

These simple 'forecast' maps may have direct relevance to many aspects of human endeavour such as food production and commodity marketing; they may be readily applied to agriculture. Use of the SOI phase system has been found to lead to improved profitability and risk management in Australian wheat cropping. By using daily data from the analogue years associated with each of the SOI phases (for example, phases in April/May) with crop simulation models, a considerable shift in potential yield could be identified. Figure 4 shows cumulative probability distributions of potential wheat yield and the large shift in yield likelihood (shift in median yield of 1,500 kg ha⁻¹) for a simulated crop grown at Goondiwindi, Australia (southern Queensland) depending on whether the SOI phase the previous austral autumn represented 'rapid fall' or 'rapid rise' patterns. Incorporation of global sea surface temperature data may further improve this simple, statistically based rainfall probability system. □

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- Ropelewski, C. F. & Halpert, M. S. *Mon. Weath. Rev.* **115**, 1606–1626 (1987).
- Ropelewski, C. F. & Halpert, M. S. *J. Clim.* **9**, 1043–1059 (1996).
- Kiladis, G. N. & Diaz, H. F. *J. Clim.* **2**, 1069–1090 (1989).
- Nicholls, N. *J. Climatol.* **5**, 553–560 (1985).
- Cane, M. A., Eshel, G. & Buckland, R. W. *Nature* **370**, 204–205 (1994).
- Hammer, G. L., McKeon, G. M., Clewett, J. F. & Woodruff, D. R. in *Proc. Conf. Agricultural Meteorology* 15–23 (Bureau of Meteorology, Melbourne, Australia, 1991).
- Hammer, G. L., Holzworth, D. P. & Stone, R. C. *Aust. J. Agric. Res.* **47**, 717–737 (1996).
- Troup, A. J. *J. R. Meteorol. Soc.* **91**, 490–506 (1965).
- McBride, J. L. & Nicholls, N. *Mon. Weath. Rev.* **111**, 1998–2004 (1983).
- Chu, P. S. *Int. J. Climatol.* **9**, 619–632 (1989).
- Gordon, N. D. *Mon. Weath. Rev.* **114**, 371–387 (1986).
- Stone, R. C. & Aulicciems, A. *Int. J. Climatol.* **12**, 625–636 (1992).
- Madden, R. A. & Julian, P. R. *J. Atmos. Sci.* **29**, 1109–1123 (1972).

- Williams, M. *Relations between the Southern Oscillation and the Troposphere over Australia* (Res. Rep. No. 6, Bureau of Meteorology Res. Centre, Melbourne, Australia, 1987).
- Stone, R. C., Nicholls, N. & Hammer, G. L. *J. Clim.* **9**, 1896–1909 (1996).
- Meinke, H., Stone, R. C. & Hammer, G. L. *Int. J. Climatol.* **16**, 783–789 (1996).
- The Global Historical Climatology Network: Long-Term Monthly Precipitation, Sea-Level Pressure, and Station Pressure Data (Environ. Sci. Div. Publ. No. 3912, Oak Ridge Natl Lab., Oak Ridge, Tennessee, 1992).
- Fraedrich, K. *Tellus* **46A**, 541–552 (1994).
- Clewett, J. F., Clarkson, N. M., Owens, D. T. & Arbrecht, D. G. *Australian Rainman: Rainfall Information for Better Management* (Dep of Primary Industries, Brisbane, Australia, 1994).

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Estimates of ozone depletion and skin cancer incidence to examine the Vienna Convention achievements

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DEPLETION of the ozone layer has been observed on a global scale¹, and is probably related to halocarbon emissions. Ozone depletion increases the biologically harmful solar ultraviolet radiation reaching the surface of the Earth, which leads to a variety of adverse effects, including an increase in the incidence of skin cancer. The 1985 Vienna Convention provided the framework for international restrictions on the production of ozone-depleting substances. The consequences of such restrictions have not yet been assessed in terms of effects avoided. Here we present a new method of estimating future excess skin cancer risks which is used to compare effects of a 'no restrictions' scenario with two restrictive scenarios specified under the Vienna Convention: the Montreal Protocol, and the much stricter Copenhagen Amendments. The no-restrictions and Montreal Protocol scenarios produce a runaway increase in skin cancer incidence, up to a quadrupling and doubling, respectively, by the year 2100. The Copenhagen Amendments scenario leads to an ozone minimum around the year 2000, and a peak relative increase in incidence of skin cancer of almost 10% occurring 60 years later. These results demonstrate the importance of the international measures agreed upon under the Vienna Convention.

The assessment model presented here integrates dynamic aspects of the full source-risk chain: from the production and emission of ozone-depleting substances through ozone depletion and increases in carcinogenic ultraviolet (UV) radiation up to increases in skin cancer incidence. The combination of predictions for changes in atmospheric ozone and effective UV with dynamic models for skin cancer induction allows full scenario studies, in contrast to earlier skin cancer risk assessments^{2,3}. Those assessments were based on the comparison of risks for two stationary situations² and did not include dynamical aspects of the skin cancer development: that is, the delay between exposure and tumour development³. We evaluated the skin cancer types: squamous cell carcinoma (SCC), basal cell carcinoma (BCC), the most frequent but least aggressive, and cutaneous malignant melanoma (CMM), the least frequent but most aggressive⁴. Present skin cancer incidence in the USA is about 2,000 per million per year⁵, and in northwest Europe an incidence of about 1,100 per million per year is estimated, using incidences given for the Netherlands⁶ as representative.

In the no restrictions (NR) scenario we assumed a continuous 3% annual increase in the production of chlorofluorocarbons (CFCs), halons and methyl chloroform, whereas in the Montreal Protocol (MP) scenario the production of five important ozone-depleting chemicals is reduced to 50% by the end of 1999, as agreed upon in 1987. In the Copenhagen Amendments (CA) scenario (1992) the production of 21 ozone-depleting chemicals is reduced to zero by the end of 1995. We assumed full global compliance with these restrictions. Unchanged human behaviour with regard to sun exposure is assumed for all scenarios.

Emitted halocarbons are transported to the stratosphere, where photochemical breakdown releases active chlorine and bromine atoms which catalyse ozone destruction. An effective chlorine loading was calculated using the method described in ref. 7. The evaluation of ozone measurements suggests that downward trends started around 1975–80, when chlorine levels were already elevated^{7,8}, suggesting that ozone depletion started after a threshold in effective chlorine loading was reached^{7,8}. Above the threshold a linear relation between chlorine loading and relative decreases in ozone columns is applied in line with a stratospheric model⁹ and observations. At very high chlorine levels ozone in the lower stratosphere is almost fully destroyed and saturation of ozone depletion is inferred¹⁰. Ozone depletion increases the solar UV-B radiation reaching the ground¹¹. We calculated the solar UV spectrum at ground level using a two-stream atmospheric UV transfer model, validated by comparison with high-resolution spectral measurements^{12,13}. The solar UV-B radiation causes a variety of adverse biological effects. The carcinogenic UV dose is ascertained by weighting of the ambient UV spectrum according to the wavelength dependence of SCC induction in mice¹⁴ with a correction for absorption differences between murine and human skin¹⁵. Skin cancer incidence in relation to age and ambient UV⁵ can be described with the dose-response relationship for SCC in

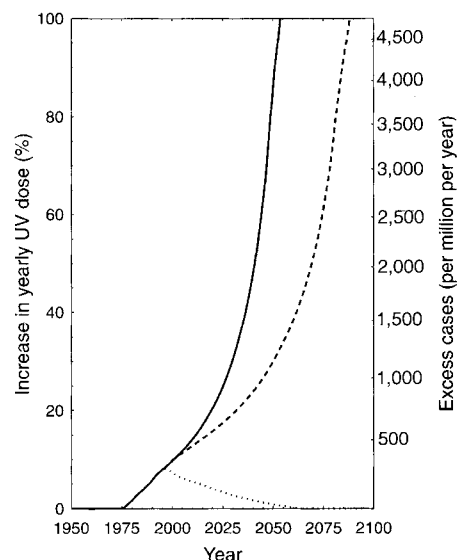


FIG. 1 Relative increase in yearly effective UV dose received at ground level at 40° North latitude (representative for the USA). The solid line represents the NR scenario, the broken line the MP scenario, and the dotted line the CA scenario. The right-hand y-axis indicates the excess skin-cancer incidence for the population in the USA (using the 1980 age distribution), which is associated with a stationary lifelong prolonged exposure at the enhanced UV level indicated on the left-hand y-axis. The relative increases in UV doses for northwest Europe (52° North) are similar. Because of the lower natural UV doses received in northwest Europe, the excess risks would be 50–55% of the excess risks for the USA. Worldwide halocarbon production and emission data were obtained from AFEAS²³ and ref. 7. We used an effective chlorine loading of 1.6 p.p.b.v. (reached in 1976) as the threshold value for ozone destruction (Cl_0). The observed 1979–91 trend in yearly averaged ozone columns of $-3.9 \pm 0.7\%$ per decade (Northern Hemisphere, mid-latitude, Dobson network), reported by the WMO¹, is used to determine the decrease in the ozone column in relation to the increase in chlorine loading: $-4.6 \pm 0.8\%$ per p.p.b.v. The natural variation in ozone over the year and the currently observed seasonal variation in ozone depletion are accounted for²⁴. A saturation in ozone depletion is inferred to occur at very high chlorine levels. Kerr¹⁰ argued that this saturation probably occurs in the Antarctic spring, when ozone values of 100 Dobson units (DU) are observed. We applied saturation levels obtained from model calculations⁹, which range at mid-latitude from ~60 DU in winter to 140 DU in summer. For our mid-latitude calculations this situation only occurs in the NR and MP protocols near the end of the next century.

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TABLE 1 Comparison of excess cases of skin cancer per million per year for the USA and northwest Europe

USA						
Year	NR	MP	CA	NR-CA	MP-CA	NR-MP
2000	36 (14–82)	36 (14–82)	31 (12–75)	5 (2–9)	5 (2–10)	0
2030	233 (98–511)	192 (81–411)	102 (32–227)	132 (43–312)	91 (30–204)	41 (13–110)
2050	705 (242–1568)	420 (170–900)	148 (48–282)	558 (161–1,322)	272 (93–615)	285 (71–724)
2070	1,890 (560–4,390)	758 (294–1,694)	122 (42–224)	1,768 (491–4,223)	636 (227–1,487)	1,132 (278–2,935)
2100	6,530 (1,766–17,871)	1,958 (627–4,616)	35 (6–93)	6,494 (1,753–17,822)	1,922 (604–4,578)	4,572 (916–13,956)
Northwest Europe						
Year	NR	MP	CA	NR-CA	MP-CA	NR-MP
2000	17 (6–41)	17 (6–41)	14 (5–37)	2 (1–4)	2 (1–4)	0
2030	121 (50–258)	103 (42–217)	58 (18–134)	62 (20–163)	44 (14–103)	18 (6–50)
2050	348 (123–904)	231 (91–474)	89 (27–173)	260 (77–743)	142 (46–328)	118 (30–386)
2070	1,017 (430–2,669)	414 (161–976)	77 (25–147)	940 (252–2,547)	338 (115–851)	602 (138–1,734)
2100	3,468 (898–11,258)	1,051 (326–2,862)	23 (4–61)	3,445 (884–11,220)	1,029 (312–2,814)	2,417 (489–8,019)

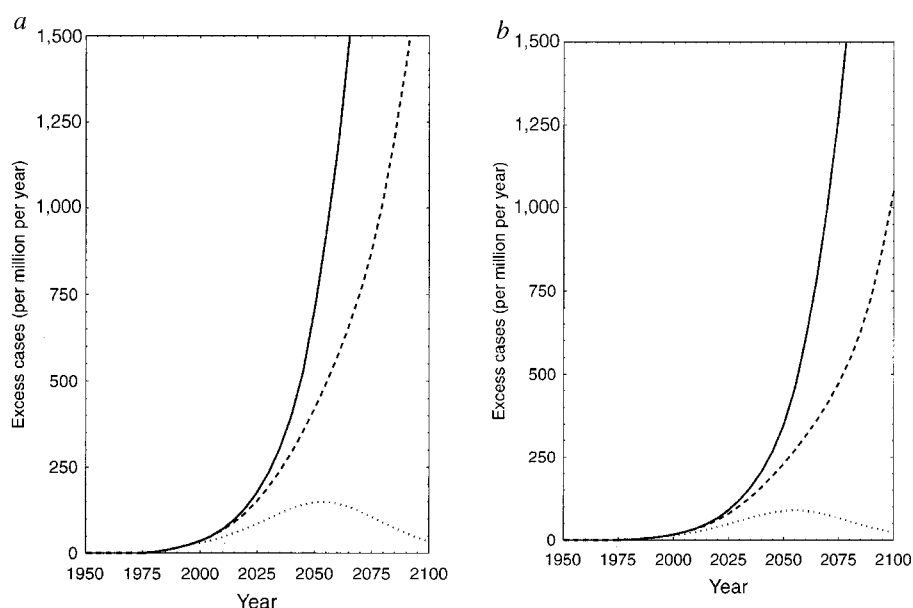
Best estimates and 95% confidence intervals are shown in parentheses. We applied a Monte Carlo-based uncertainty analysis to obtain an indication of the confidence intervals for the projections.

mice, with adjusted parameters^{16,17}. SCC is related to the lifelong accumulated UV dose^{5,16}, but for BCC and CMM epidemiological findings indicate that UV exposure acts mainly in the early stages of tumour development^{18,19}. These differences in the timing of UV-driven processes are incorporated into the present modelling. On the basis of this model, extrapolation of incidences of BCC and SCC from the USA to the Netherlands agreed within 11% with reported incidences⁶; the CMM incidence, however, deviates by 45%, indicating a larger uncertainty for the CMM projections.

The relative increase in yearly effective UV dose at 40° North latitude in the USA is shown in Fig. 1. Results from a previous risk

assessment², which provided stationary excess risks associated with the ozone deficit reported between 1979 and 1992, are in agreement with the stationary risks for that period shown in Fig. 1. Figure 2 shows the excess risk if the dynamical aspects of the skin cancer models are included. Comparison of Figures 1 and 2 indicates a 60-year delay between the exposure and risk peaks in the CA scenario. Assuming a population of 225 million for the USA we estimated the excess skin cancer cases due to ozone depletion around the year 2100 at 1,500,000 (+325%), 440,000 (+100%) and 8,000 (+2%) per year for the NR, MP and CA scenarios, respectively. For northwest Europe, these numbers are

FIG. 2 Excess incidences calculated for the USA (a) and northwestern Europe (b) populations, incorporating the delay between exposure and the occurrence of tumours. The solid line represents the NR scenario, the broken line the MP scenario, and the dotted line the CA scenario. The number of SCC in a birth cohort is directly proportional to $\Phi(a)^c \times a^{d-c}$, where $\Phi(a)$ is the cumulative UV dose up to age a , and $c = 2.5 \pm 0.7$ and $d = 6.6 \pm 0.4$ and are parameters derived from epidemiology^{16,17}. For BCC, epidemiology and data on genetic changes in the tumours²⁵ also implicate solar UV exposure as a cause. As found in SCC, the p53 tumour-suppressor gene in a majority of BCCs has UV-like mutations²⁵. In experiments, these p53 mutations are found to be early events and detectable in microscopic foci of cells before the occurrence of the skin tumours²⁶. However, the accumulated UV exposure in the 10–20 years directly preceding the detection of the tumour appears unimportant^{18,19}. Thus the evidence suggests that UV exposure acts mainly in the early stages of BCC development. Similarly, exposure to the sun in youth appears to be an important risk factor for CMM later in life⁴. The yield of BCC and CMM at age a is proportional to $\sum D(x) \times \Phi(x)^{c-1} \times (a-x)^{d-c}$ (ref. 27), where $D(x)$ is the dose received



in the year at age x , and the summation is over all years x , from age 0 up to age a . For BCC, $c = 1.4 \pm 0.4$, $d = 4.9 \pm 0.6$ (ref. 21) and for CMM, $c = 0.6 \pm 0.4$, $d = 4.7 \pm 1.0$ (calculated from ref. 28).

550,000 (+315%), 170,000 (+95%) and 4,000 (+2%) per year, respectively, assuming a population of 160 million (including Benelux, Denmark, Germany and Great Britain). Relative increases in incidence for the year 2050 are 35%, 21% and 7.5% for the NR, MP and CA scenarios. The difference between the effects calculated for the NR and CA scenarios is a measure of what was achieved by the Vienna Convention. The difference between the effects in the MP and CA scenarios illustrates the benefit of the more stringent restrictions.

Clearly uncertainties are involved in predicting future skin cancer risks accurately. An integrative modelling approach²⁰ allows an evaluation of accuracies in relation to uncertainties in the model parameters. In a Monte Carlo procedure, we varied parameter values to determine the confidence intervals for the risk projections; see Table 1. Although the confidence intervals are wide (factors of 5–10), the differences between the three scenarios (last three columns in Table 1) remain significant, because the parameters dominating the uncertainties produce highly correlated results for the three scenarios. The CMMs projections must, however, be regarded with some reservations. We used a carcinogenic dose which is in line with known DNA-damaging effects of UV in various cell types. CMM have, however, been induced in fish with an unexpectedly high efficacy in the UV-A²¹. If this holds for humans too, then CMM would not react appreciably to an ozone depletion. This would hardly affect the projected excess cases because CMM contributes 1–4%. However, the excess mortality risk would be reduced from about 2% of the excess incidences to 1–1.4%. Other systematic changes in UV exposure as a result of changes in human behaviour, and changes in atmospheric parameters, like tropospheric ozone, aerosol and cloud cover²², could influence future skin cancer risks. However, these possible modifications would not influence the significance of the differences between the scenarios. It is conceivable that the results for skin cancer will also be representative for some of the other possibly even more important adverse effects. Monitoring of ozone and UV levels remains necessary to assess the success rate of implementing the policy agreements.

Our estimates of the excess skin cancer cases avoided should be considered conservative for the following three reasons. First, the increase in yearly production assumed in the NR scenario is lower than the annual increases observed in the mid-1980s. Second, we did not include the higher ozone trends in the most recent years which could be influenced by the volcanic eruption of the Mount Pinatubo in 1991. Using the 1979–94 trends would increase the excess incidence and mortality estimates by 20–40%. Third, the considered populations will age in the next 50 years, implying increasing excess risks caused by ozone depletion. An analysis for the Netherlands has shown a 60% increase by the year 2040 (ref. 20).

Even in the CA scenario the calculated number of excess cases caused by ozone depletion exceeds 33,000 per year in the USA around the year 2050 and 14,000 per year in northwest Europe. The projections presented demonstrate, however, the important improvements achieved if the measures agreed upon under the Vienna Convention are fully implemented. □

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1. World Meteorological Organization in *Scientific Assessment of Ozone Depletion: 1994 Global Ozone Research and Monitoring Project* WMO Rep. 37 (WMO, Geneva, 1995).
2. Madronich, S. & de Groot, F. R. *Nature* **366**, 23 (1993).
3. Krieger, A., Armstrong, B. K. & McMichael, A. J. *Nature* **368**, 594 (1994).
4. Holman, C. D. J. & Armstrong, B. K. *J. Natl Cancer Inst.* **73**, 75–82 (1984).
5. Scotto, J. & Fears, T. R. *Incidence in Non-melanoma Skin Cancer in the United States* NIH Publication 82-2433 (National Institutes of Health, Washington DC, 1981).
6. Health Council of the Netherlands *UV Radiation from Sunlight, The Hague 1994/05E* (Health Council of the Netherlands, The Hague, 1994).
7. Daniel, J. S., Solomon, S. & Albritton, D. L. *J. Geophys. Res.* **D 100**, 1271–1285 (1995).
8. World Meteorological Organization in *Scientific Assessment of Ozone Depletion: 1991 Global Ozone Research and Monitoring Project* WMO Rep. 25 (WMO, Geneva, 1992).
9. Velders, G. J. M. *Scenario Study of the Effects of CFC, HCFC and HFC Emissions on Stratospheric Ozone* Report 722201006 (RIVM, Bilthoven, the Netherlands, 1995).
10. Kerr, R. A. *Science* **270**, 376 (1995).
11. McKenzie, R. L. et al. in *Global Ozone Research and Monitoring Project* WMO Rep. 37 Chapter 9 (WMO, Geneva, 1995).
12. Slaper, H., Reinen, H. A. J. M., Blumthaler, M., Huber, M. & Kuik, F. *Geophys. Res. Lett.* **22**, 2721–2724 (1995).

13. Bordewijk, J. A., Slaper, H., Reinen, H. A. J. M. & Schlamann, E. *Geophys. Res. Lett.* **22**, 2151–2154 (1995).
14. De Groot, F. R. et al. *Cancer Res.* **53**, 53–60 (1993).
15. De Groot, F. R. & van der Leun, J. C. *Health Phys.* **67**, 319–325 (1994).
16. Slaper, H., Schothorst, A. A. & van der Leun, J. C. *Photodermatology* **3**, 271–283 (1986).
17. De Groot, F. R. & Forbes, P. D. *BioEssays* **17**, 651–660 (1995).
18. Vitasa, B. C. et al. *Cancer* **65**, 2611–2617 (1990).
19. Gallagher, R. P. et al. *Arch. Dermatol.* **131**, 157–163 (1995).
20. Slaper, H., den Elzen, M. G. J., van der Woerd, H. J. & de Greef, J. in *Ozone Depletion and Skin Cancer Incidence: An Integrated Modeling Approach* Report 749202001 (RIVM, Bilthoven, the Netherlands, 1992).
21. Setlow, R. B., Grist, E., Thompson, K. & Woodhead, A. *Proc. Natl Acad. Sci. USA* **90**, 6666–6670 (1993).
22. Lubin, D. & Jensen, E. H. *Nature* **377**, 710–713 (1995).
23. *Alternative Fluorocarbon Environmental Acceptability Study Production, Sales and Atmospheric Release of Fluorocarbons through 1992* (Grant Thornton, Washington DC, 1993).
24. de Winter, R. in *Depletion and Natural Variability of the Ozone Layer from TOMS Observation* Report 722201005 (RIVM, Bilthoven, the Netherlands, 1995).
25. Ziegler, A. et al. *Proc. Natl Acad. Sci. USA* **90**, 4216–4220 (1993).
26. Berg, R. J. W. et al. *Proc. Natl Acad. Sci. USA* **93**, 274–278 (1996).
27. Slaper, H. thesis, Utrecht University (1987).
28. Longstreth, J. D. (ed.) *Ultraviolet Radiation and Melanoma* UA-EPA 400/1-87/001D (US Environmental Protection Agency, Washington DC, 1987).

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Functional imaging of an illusion of pain

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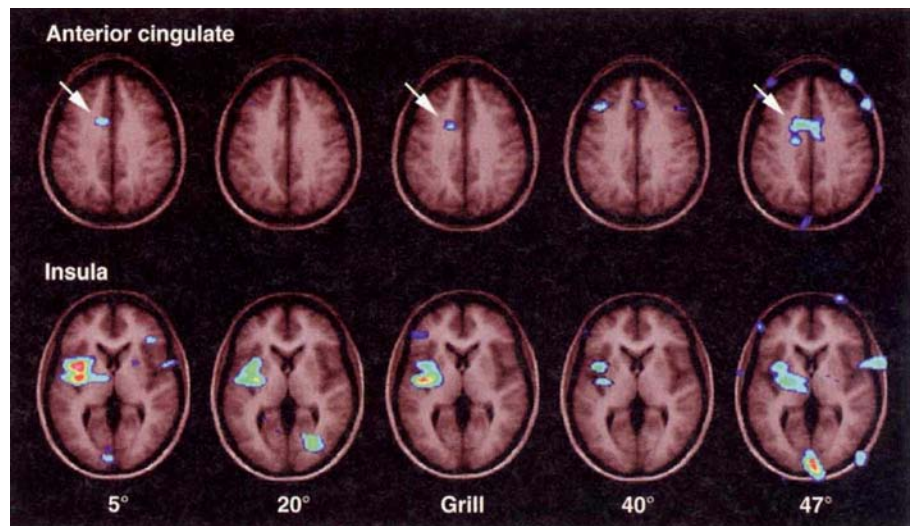
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TOUCHING warm and cool bars that are spatially interlaced produces a painful burning sensation resembling that caused by intense, noxious cold. We demonstrated previously that this thermal grill illusion can be explained as an unmasking phenomenon that reveals the central inhibition of pain by thermosensory integration¹. In order to localize this unmasking in the human brain, we have used positron emission tomography (PET) to compare the cortical activation patterns evoked by the thermal grill and by cool, warm, noxious cold and noxious heat stimuli. The thermal grill illusion produces activation in the anterior cingulate cortex, whereas its component warm and cool stimuli do not. This area is also activated by noxious heat or cold. Thus, increased activity in the anterior cingulate cortex appears to be selectively associated with the perception of thermal pain. Disruption of thermosensory and pain integration may account for the central pain syndrome that can occur after stroke damage.

The possibility of fundamental interactions between the sensations of pain and temperature is suggested by the intimate anatomical relationship and functional overlap of their ascending pathways in the central nervous system². In particular, there is evidence indicating that cold inhibits pain processing centrally. Peripheral cold stimulation reduces pain evoked by electrical nerve stimulation³, and cold ambient temperatures produce behavioural antinociception⁴. A peripheral nerve block of A-fibre conduction eliminating cold sensibility produces cold allodynia, in which an innocuous cool stimulus elicits burning pain at temperatures up to 24 °C, well above the usual 15 °C, indicating that cold-specific Aδ afferent activity normally inhibits a cold-sensitive C-fibre nociceptive channel^{5–9}.

FIG. 1 Normalized axial 31-slice PET images through the anterior cingulate cortex ($z = 38$; ref. 30) and the mid/anterior insular cortex ($z = 3$), showing the significant rCBF activation for each stimulus condition compared with the neutral (34°C) condition (t values above 2.3 are highlighted). Left is contralateral to the stimulus.



Like other sensory systems, integration between ascending nociceptive and thermoreceptive sensory channels is revealed strikingly by an illusion. In the thermal grill illusion, a spatially unusual presentation of innocuous warm (40°C) and cool (20°C) temperatures produces a painful heat sensation that resembles the burn of cold pain. We have demonstrated psychophysically that this is a robust illusion, and we showed with a simple quantitative physiological model that it can be explained as an unmasking phenomenon resulting from central disinhibition of a polymodal nociceptive spinothalamic channel (lamina I HPC cells) by reduction of the activity in an innocuous thermoreceptive (cold-specific) spinothalamic channel (lamina I COLD cells)¹. We validated this model by confirming its prediction that a noxious cold stimulus of 10°C elicits a pain rating corresponding to that produced by the thermal grill illusion. The same model can explain the cold allodynia produced by peripheral nerve A fibre block.

The thermal grill provides a unique tool to investigate the central basis of pain perception. Here we investigate whether particular cortical regions might be selectively activated in association with the illusion of pain produced by the thermal grill. We used PET imaging of regional cerebral blood flow (rCBF) as a measure of neuronal activity in humans and compared the pattern of cortical activation evoked by the thermal grill with the patterns produced individually by its constituent innocuous temperatures. Based on the concept that this illusion is an unmasking phenomenon related to the inhibition of pain by cold, we predicted that the thermal grill would produce a pattern of cerebral activity resembling that produced by noxious cold. Finally we considered that unmasked activity might reveal loci at which the cold-induced inhibition of pain normally occurs.

We measured rCBF under six different conditions of thermal stimulation (see Methods): baseline (34°C), 'warm' (40°C), 'cool' (20°C), 'heat pain' (47°C), 'cold pain' (5°C), and 'grill' (interlaced 40°C and 20°C bars). Localized rCBF increases over the baseline condition were measured in four contralateral cortical regions of interest identified by previous PET studies of heat pain: pericentral or primary somatosensory (S1), lateral opercular or secondary somatosensory (S2), mid/anterior insular and anterior cingulate cortices^{10–13}.

Because all thermal stimuli were applied with the same thermometer on the hand, the results obtained with cool, warm, heat pain and cold pain are intrinsically interesting compared with other PET studies that used different methods (to be described in detail later¹⁴). Briefly, we confirmed significant rCBF increases with heat pain in all four cortical regions of interest (Table 1)^{10–13}. Cold pain activated all these areas as well, although the rCBF increase in S1 was less (below criterion), which closely resembles the findings in

another PET study¹³. These observations are consistent with the inference that discriminative and motivational aspects of thermal pain common to both noxious heat and noxious cold are processed in these cortical regions. The cortical activation patterns observed with innocuous cool and warm are novel¹³. Cool produced significant activation only in the mid/anterior insula and S2, whereas warm activated the insula, S1 and, more weakly (below criterion), S2. A direct comparison of cool and warm indicated significant differences in S1 and S2, supporting the inference that these submodalities in pain are processed differently.

The mid/anterior insula is the only cortical region that was strongly activated by all thermal stimuli, whether noxious or innocuous (Table 1 and Fig. 1). This result corroborates the integral involvement of mid/anterior insular cortex in both pain and temperature sensibilities (see below), and it is consistent with the proposed involvement of this region in a general sense of the physiological condition of the body tissues². In contrast, the anterior cingulate cortex is the only cortical region activated uniquely by the noxious stimuli (heat pain and cold pain) and not at all by the innocuous thermal stimuli. Direct subtractions of warm from noxious heat and of cool from noxious cold show activation only in the anterior cingulate. This indicates that an area within the functionally complex anterior cingulate cortex has a highly selective role in pain processing, consistent with an involvement in the characteristic emotional/motivational component (unpleasantness and urgency) of pain^{10,12,13,15}.

The thermal grill produced significant rCBF increases in the mid/anterior insula, in S2 and in the anterior cingulate, and also weaker activation (below criterion) in S1. This pattern of cerebral activation is virtually identical to that produced by the noxious cold stimulus. Direct comparison with the cold pain condition showed no statistically significant differences, whereas comparison of either the thermal grill or cold pain with the heat pain condition showed a significant difference in S1. This result directly supports the hypothesis that the painful heat sensation elicited by the thermal grill resembles the burn of cold pain.

The thermal grill caused significant cortical activation in one region, the anterior cingulate cortex, that is not activated by either of its constituent cool or warm stimuli. This observation directly associates the perception of pain produced by the thermal grill illusion with activation of a region of cortex that is normally activated selectively by noxious but not innocuous thermal stimuli. This result confirms the special role of the anterior cingulate cortex in pain, and it indicates that the activation of this cortical region is an integral component of the neurobiological basis of the thermal grill illusion of pain.

Both of these results (that is, confirmation of the similarity to cold pain and the activation of anterior cingulate cortex) are

consistent with the simple hypothesis that the thermal grill illusion is an unmasking phenomenon, in which the simultaneous presentation of cool and warm disinhibits, or unmasks, activity in an ascending cold-sensitive polymodal nociceptive channel. The cortical locus of unmasked activity is in the anterior cingulate. The central basis for the thermal grill illusion could be more complex, but the available evidence is incompatible with an alternative hypothesis of sensory fusion or with the symmetrical possibility of warm-induced masking, as discussed earlier^{1,7}.

We suggest the following explanation for the cerebral activation produced by thermal grill illusion. The cool (20 °C) bars activate only lamina I COLD cells and lamina I HPC cells. COLD cell activity normally exceeds HPC cell activity at innocuous cold temperatures (above ~15 °C), but the interlaced warm (40 °C) bars in the thermal grill decrease COLD cell activity by half, without affecting HPC or any other spinothalamic neurons^{1,16–18}. This reduction can effectively unmask ascending HPC activity by disinhibition at thalamocortical levels¹. In primates, lamina I neurons project via the lateral spinothalamic tract to two main sites: a lateral thalamic relay nucleus (VMpo) that projects topographically to the mid/anterior insular cortex^{16,19}, and a medial thalamic area (MDvc) that projects to the anterior cingulate cortex²⁰. The lateral pathway conveys both thermoreceptive and nociceptive activity¹⁶, which provides a basis for our PET imaging observation that the mid/anterior insula is the only cortical region activated by both innocuous and noxious thermal stimuli. Our finding that noxious cold and noxious heat selectively activate the anterior cingulate cortex indicates that the medial lamina I pathway conveys HPC activity (albeit probably converging with other inputs). The activation of anterior cingulate that is unmasked by the thermal grill is consistent with our proposal^{1,16} that COLD activity ascending in the lateral lamina I spino-thalamo-cortical pathway to the insula normally inhibits cold-evoked HPC activity in the medial lamina I pathway to the anterior cingulate. Thus, the present results suggest that the fundamental integration between pain and temperature sensations revealed by the thermal grill illusion, that is, the cold-induced inhibition of pain, may involve interactions between these two pathways²¹.

Finally, these results are immediately relevant to the clinical syndrome of central (post-stroke) pain^{1,16}. Analogous to the thermal grill illusion, such patients experience "burning, ice-like" pain and cold allodynia that is correlated with dysfunction of innocuous thermal sensibility and a paradoxical hypalgesia^{22–24}. Although different forms of central pain may exist, this syndrome is nearly always associated with lesions involving the lateral lamina

TABLE 1 Values of *t* for peaks with different stimulus conditions

Location	5°	20°	Grill illusion (20° + 40°)	40°	47°
S1 (−30, −16, 35)	2.30*	<1.00	1.97*	3.58†	3.83†
S2 (−55, −8, 13)	4.0†	3.74†	2.92†	2.08*	3.43†
Insula (−37, −9, 3) (−37, 5, 3)	4.50†	3.75†	4.17†	3.33†	3.37†
Anterior cingulate (−10, 7, 38)	3.00†	<1.00	2.77†	<1.00	3.22†

Stereotaxic coordinates (x, y, z; that is, medial–lateral, anterior–posterior, superior–inferior)³⁰ are given for each location. The two peaks observed in the insula were not spatially statistically separable.

* Statistical trend ($P < 0.05$ if uncorrected for multiple comparisons).

† $P < 0.05$ ($t_x = 2.51$, two-tailed, corrected for 4 multiple comparisons).

I spino-thalamo-cortical pathway, in particular posterolateral thalamus and subinsular white matter, and never with lesions in medial thalamus^{22–25}. Our observations suggest that such lesions affecting COLD channel activity could unmask cold-induced activation of anterior cingulate cortex associated with the burn of cold pain, consistent with the essence of the original, prescient conjecture of Head and Holmes²⁶. □

Methods

We measured rCBF during twelve 40-s scans made in each of 11 normal right-handed volunteers (3 female, 8 male; mean age 34 years) with the ECAT 951/31 (Siemens) tomograph following bolus injections of 45 mCi of $H_2^{15}O$ (ref. 27). Thermal stimuli were applied to the palmar surface of the right hand with a 20 × 14 cm thermal device consisting of 15 parallel silver bars¹. Each volunteer gave separate magnitude estimations of the maximum intensity of heat, cold and pain felt during each stimulus presentation. As in our earlier study¹, innocuous thermal stimuli were rated as simply cool or warm and only rarely painful; the noxious stimuli were consistently rated as painfully hot or painfully cold, and the thermal grill was rated as significantly painful, as slightly cool (half as cold as 20 °C alone) and as hotter than 40 °C alone. To maximize sensitivity, stimuli were timed to produce a peak sensation about 5 s before the maximum radioactivity uptake in the brain (expected at 15 s after bolus injection). The six different 1 min stimuli were presented twice each, in counterbalanced order. The two scans of each condition were averaged, filtered (18 mm), normalized for global changes, co-registered with individual magnetic resonance images (MRIs), and transformed into stereotaxic coordinate space^{12,28}. The PET volume for the neutral condition was then subtracted (voxel by voxel) from each active thermal condition, the subtraction images were averaged across volunteers, converted to a *t* statistic volume²⁹, and then superimposed on an averaged MRI volume. Significant rCBF activation in the four regions of interest occurred at $t > 2.51$, yielding $P < 0.05$ when corrected for multiple comparisons.

20. Craig, A. D. & Zhang, E. -T. *Soc. Neurosci. Abstr.* **22**, 11 (1996).

21. Van Hoesen, G. W., Morecraft, R. J. & Vogt, B. A. in *Neurobiology of Cingulate Cortex and Limbic Thalamus* (eds Vogt, B. A. & Gabriel, M.) 249–284 (Birkhäuser, Basel, 1996).

22. Boivie, J., Leijon, G. & Johansson, I. *Pain* **37**, 173–185 (1989).

23. Boivie, J. & Leijon, G. in *Pain and Central Nervous System Disease: The Central Pain Syndromes* (ed. Casey, K. L.) 65–76 (Raven, New York, 1991).

24. Schmähmann, J. D. & Leifer, D. *Arch. Neurol.* **49**, 1032–1037 (1992).

25. Bogousslavsky, J., Regli, F. & Uske, A. *Neurology* **38**, 837–848 (1988).

26. Head, H. & Holmes, G. *Brain* **34**, 102–254 (1911).

27. Reiman, E. M. et al. *N. Engl. J. Med.* **334**, 752–758 (1996).

28. Evans, A. C., Marrett, S., Torrescorzo, J., Ku, S. & Collins, L. J. *Cereb. Blood Flow Metab.* **11**, A69–A78 (1991).

29. Worsley, K. J., Evans, A. C., Marrett, S. & Neelin, P. J. *Cereb. Blood Flow Metab.* **12**, 900–918 (1992).

30. Talairach, J. & Tournoux, P. *Co-planar Stereotaxic Atlas of the Human Brain* (Thieme, New York, 1988).

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- Craig, A. D. & Bushnell, M. C. *Science* **265**, 252–255 (1994).
- Craig, A. D. in *Somesthesia and the Neurobiology of the Somatosensory Cortex* (eds Franzen, O., Johansson, R. & Terenius, L.) 27–39 (Birkhäuser, Basel, 1996).
- Bini, G., Cruccu, G., Hagbarth, K. -E., Schady, W. & Torebjörk, E. *Pain* **18**, 239–248 (1984).
- Osgood, P. F. et al. *Brain Res.* **507**, 11–16 (1990).
- Fruhstorfer, H. *Pain* **20**, 355–361 (1984).
- LaMotte, R. H. & Thalhacker, J. G. *Brain Res.* **244**, 279–287 (1982).
- Yarnitsky, D. & Ochoa, J. L. *Brain* **113**, 893–902 (1990).
- Wahren, L. K., Torebjörk, E. & Jörum, E. *Pain* **38**, 313–319 (1989).
- Ochoa, J. L. & Yarnitsky, D. *Brain* **117**, 185–197 (1996).
- Talbot, J. D. et al. *Science* **251**, 1355–1358 (1991).
- Jones, A. K. P., Brown, W. D., Friston, K. J., K. J., Qi, L. Y. & Frackowiak, R. S. J. *Proc. R. Soc. Lond.* **244**, 39–44 (1991).
- Coghil, R. C. et al. *J. Neurosci.* **14**, 4095–4108 (1994).
- Casey, K. L., Minoshima, S., Morrow, T. J. & Koeppe, R. A. *J. Neurophysiol.* **76**, 571–581 (1996).
- Bushnell, M. C., Craig, A. D., Reiman, E. M., Yun, L. -S. & Evans, A. *Soc. Neurosci. Abstr.* **21**, 1637 (1995).
- Sikes, R. W. & Vogt, B. A. *J. Neurophysiol.* **68**, 1720–1732 (1992).
- Craig, A. D., Bushnell, M. C., Zhang, E. -T. & Blomqvist, A. *Nature* **372**, 770–773 (1994).
- Kenshalo, D. R. Jr, Leonard, R. B., Chung, J. M. & Willis, W. D. *Pain* **12**, 141–152 (1982).
- Craig, A. D. & Dostrovsky, J. O. *Exp. Brain Res.* **85**, 470–474 (1991).
- Craig, A. D., Krout, K. & Zhang, E. -T. *Soc. Neurosci. Abstr.* **21**, 1165 (1995).

Regulation of an extrathymic T-cell development pathway by oncostatin M

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Most of the T lymphocytes that populate the immune system develop in the thymus before its involution during late adolescence. Therefore, subsequent losses in T cells caused by HIV infection¹, chemotherapy² or age-related factors³ can greatly diminish immune responses to new antigenic challenge. Here we report the discovery of a thymus-independent pathway of T-cell development that may provide help for T-cell immunodeficiency. We show that expression of an oncostatin M transgene⁴ in the early T lineage stimulates a dramatic accumulation of immature and mature T cells in lymph nodes. A functional thymus is not required for this effect as reconstitution of *nu/nu* mice with transgenic bone marrow stimulated a 500-fold increase in Thy-1⁺ lymph node cells and restored immune responsiveness to allogeneic mouse melanoma cells. This lymphopoietic pathway is not unique to transgenic mice because administration of oncostatin M protein produced a similar response in non-transgenic mice. These results identify a new pathway of T-cell development and a potential treatment for T-cell immunodeficiency with oncostatin M.

Oncostatin M (OM) is a member of the interleukin-6 (IL-6) subfamily of cytokines that is expressed by haematopoietic tissues and cells^{5,6}. When the p56^{lck} proximal promoter was used to express the bovine oncostatin M (OM) gene in the early T lineage⁴, there was a dramatic accumulation of lymph node cells that resembled thymocytes, that is, with an antigen profile Thy-1⁺CD4⁺CD8^α⁺8β⁺CD3^{lo}HSA⁺. By 10 weeks of age, the cellularity of the LckOM transgenic mesenteric node increased 20-fold relative to controls, with the CD4⁺CD8⁺ cell frequency exceeding 80%, a level that approximates a normal thymus (Fig. 1a). These CD4⁺CD8⁺ node cells expressed a repertoire of T-cell antigen receptor (TCR) Vβ molecules that was as diverse as CD4⁺CD8⁺ thymocytes, indicative of their polyclonal origin (Fig. 1b). Normal thymus contains Thy1⁺CD25⁺HSA⁺ cells that are CD4⁺CD8⁺CD3⁺B220⁺, a stage of development that precedes the onset of TCR β-chain gene rearrangement⁷. As shown in Fig. 1c, the LckOM mesenteric node contained similar types of cells. Further evidence that these are related to the early-stage thymocytes that express a pre-TCR complex was the detection of pTα RNA⁸ in transgenic nodes but not control nodes (Fig. 1d). These results indicate that OM stimulates T-cell development in the lymph nodes of transgenic mice.

To test whether this lymphopoietic activity is thymus-independent, we reconstituted lethally irradiated B6 nude mice with Thy1-depleted B6 bone marrow prepared from either LckOM transgenic or non-transgenic mice. After sixteen weeks, the mesenteric nodes in mice receiving LckOM bone marrow contained 500 times more Thy1⁺ cells than control animals (Table 1); 77% of these cells were CD4⁺CD8⁺ and the other 23% were either CD4⁺CD8⁺ or CD4⁺CD8⁺. To investigate their immunocompetence, we tested the ability of these mice to reject tumours: allogeneic H-2D^b clone 62 mouse melanoma cells⁹ were implanted in both groups of nude animals (H-2D^b) and allowed to grow for 10 days. Tumours in mice reconstituted with transgenic cells were only 10% of the volume as those in untreated B6 nude mice or in mice grafted with control B6 bone marrow (Fig. 2). Thus, the T cells

produced in LckOM-reconstituted nude mice were functional in rejecting allogeneic tumour-cell growth.

The thymus independence of this developmental pathway was also demonstrated in thymectomized mice, of which 6 animals receiving LckOM bone marrow developed CD4⁺CD8⁺ node cells, whereas thymectomized mice receiving control bone marrow did not (data not shown). To determine whether this response was

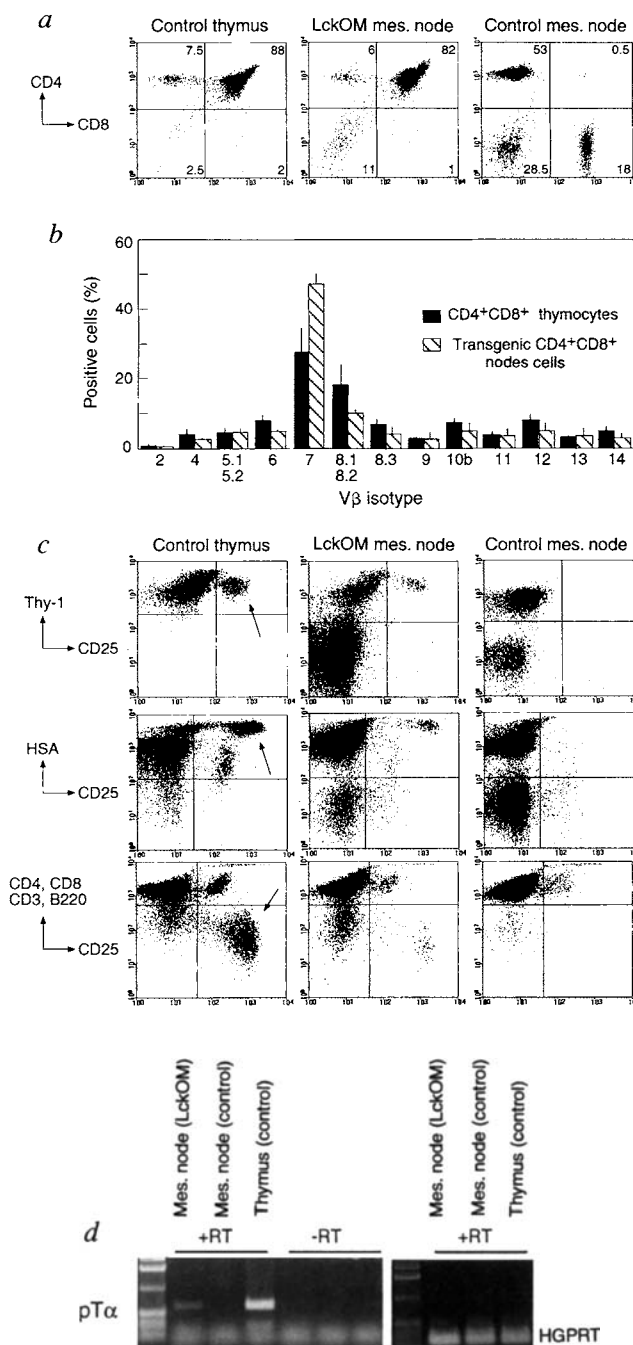


FIG. 1 T-cell lymphopoiesis in the mesenteric node (mes. node) of LckOM transgenic mice. **a**, CD4 and CD8 expression on cells isolated from the indicated tissues of 10-week-old mice. **b**, Relative expression of the indicated Vβ isoforms in non-transgenic CD4⁺CD8⁺ thymocytes and LckOM CD4⁺CD8⁺ mesenteric node cells. **c**, Detection of early T-cell precursor cells (Thy1⁺CD25⁺HSA⁺CD4⁺CD8⁺CD3⁺B220⁺) in normal thymus (arrows) and LckOM mesenteric node. **d**, Detection of pTα RNA in LckOM mesenteric node. Cellular RNA was isolated from the indicated tissues, selectively treated with reverse transcriptase (RT), and then subjected to PCR using primers specific for the mouse pTα and HGPRT (hypoxanthine-guanine phosphoribosyltransferase) genes⁸.

TABLE 1 T-cell development in *nu/nu* mouse mesenteric node following reconstitution with LckOM bone marrow*

Donor bone marrow	Total cells†	B cells‡	T cells§	CD4 ⁺ CD8 ⁻	CD4 ⁺	CD8 ⁺
B6	$3 \times 10^5 (\pm 1.3 \times 10^5)$	$2.8 \times 10^5 (\pm 1.2 \times 10^5)$	$2.4 \times 10^4 (\pm 1.1 \times 10^4)$	1.6×10^3	1.1×10^4	1.2×10^4
LckOM	$3.8 \times 10^7 (\pm 5 \times 10^6)$	$2.5 \times 10^7 (\pm 6 \times 10^6)$	$1.2 \times 10^7 (\pm 7 \times 10^6)$	9.4×10^6	1.6×10^6	1.1×10^6
Fold difference	126×	89×	500×	5,875×	145×	91×

* Measured 14 weeks after bone marrow transplantation

† Total lymphocytes ($\pm$ s.d.) isolated from 4 mesenteric nodes.‡ B220⁺Thy1⁻ cells.§ Total Thy1⁺ cells.

peculiar to transgenic cells, we reconstituted thymectomized B6 mice with a 1:1 mixture of bone marrow taken from transgenic and Thy1.1 congenic mice. Within 6 weeks, the mesenteric nodes of three thymectomized mice receiving LckOM (Thy1.2) + B6Thy1.1 bone marrow contained 5 times more cells than that in the control animals, and nearly 30% of these cells were CD4⁺CD8⁺ (Fig. 3a). Moreover, 30% of these CD4⁺CD8⁺ cells were derived from the non-transgenic Thy1.1⁺ bone marrow (Fig. 3c), as were 25–30% of the single-positive CD4⁺ (Fig. 3e) and CD8⁺ cells (data not shown). This result indicates that normal bone marrow cells can respond to OM and support T-cell development in non-transgenic lymph nodes.

Further evidence indicated that OM protein can regulate T-cell development in nude mice. When mice were treated with a sublethal dose of radiation, followed by daily injections of OM protein (10 μ g per day), the combined frequencies of CD4⁺CD8⁺, CD4⁺CD8⁻ and CD4⁻CD8⁺ T cells increased ~5-fold relative to non-treated mice, and 2.5-fold over irradiated mice treated with PBS (Fig. 4). This result strengthens the hypothesis that T-cell development can occur in the mesenteric nodes of non-transgenic mice and that OM has a direct effect on this process.

OM belongs to the IL-6 subfamily of cytokines that share a four- α -helical-bundle structure and heteromeric receptor complexes containing the signal-transducing subunit gp130 (refs 5, 10). Evidence that members of this family regulate early stages of a haematopoiesis is quite strong. For instance, disruption of the IL-6 (ref. 11) and LIF (ref. 12) genes diminish haematopoietic stem-cell numbers and the rate of thymocyte proliferation. Overexpression of LIF also stimulates peripheral CD4⁺CD8⁺ cell accumulation¹³, and elimination of gp130 dramatically reduces haematopoiesis and thymus cellularity¹⁴. OM is induced through the JAK-Stat5 pathway⁶ and regulates a broad range of cellular responses *in vivo* and *in vitro*^{4,5,15}. On the basis of our results, we suggest that OM can regulate an extrathymic pathway of T-cell development in lymph nodes. In contrast to the gut-derived T cells that express a CD8 $\alpha\alpha$ homodimer¹⁶, the immature T cells described here appear to be indistinguishable from normal thymocytes (CD8 $\alpha\beta$).

Previously, the appearance of CD4⁺CD8⁺ lymph-node cells was thought to represent only thymus emigrants, but our finding that their levels can be modulated in thymectomized and nude mice argues against this. Questions arise concerning the intercellular signals that regulate this phenomenon and whether the mechanisms controlling TCR expression and function are similar to those that occur in thymus⁷. The dramatic amplification of this lymphopoietic pathway in LckOM transgenic mice provides a useful model for investigating the properties of extrathymic T-cell development. The decline in T-cell-dependent immunity associated with advanced age and various immunodeficiencies is usually considered to be irreversible after thymus involution. Here we have shown that an alternative pathway can be used to repopulate immunodeficient mice with T cells. The ability of oncostatin M to stimulate this pathway may be beneficial for treating T-cell immunodeficiency. □

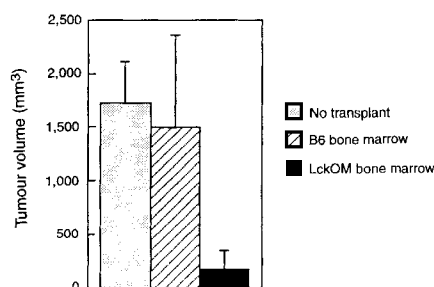


FIG. 2 Rejection of tumour growth in LckOM-reconstituted nude mice. B6 nude mice were implanted with 1×10^7 allogeneic (H-2D^b) clone 62 mouse melanoma cells⁹ 16 weeks after receiving H-2D^b bone marrow from either transgenic or non-transgenic B6 mice. Tumour volumes in each group ($n = 4$) were measured ten days later. The level of T-cell repopulation is indicated in Table 1.

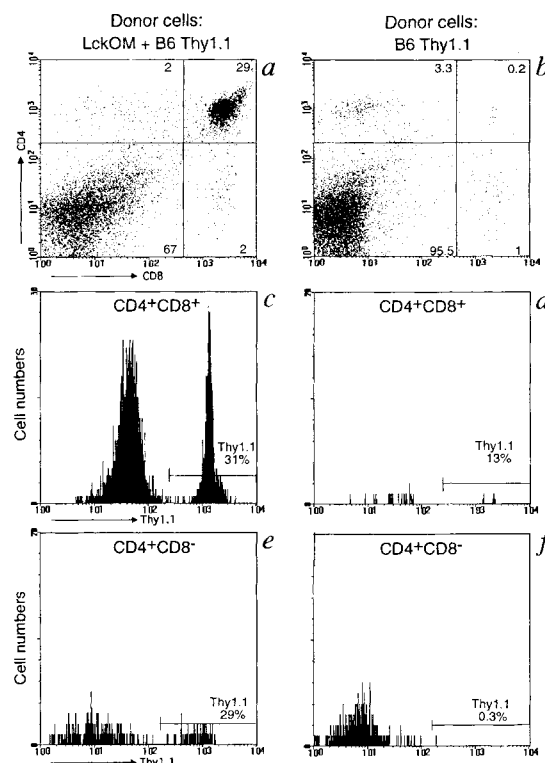
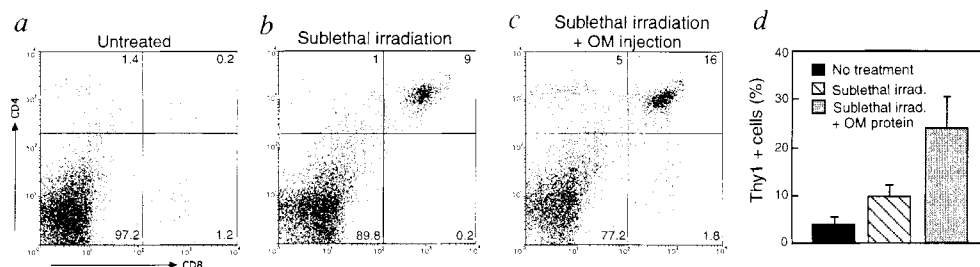


FIG. 3 Non-transgenic bone marrow can support extrathymic T-cell development in mesenteric node. Lethally irradiated, thymectomized C57BL/6 (Thy1.2) mice were injected with a 1:1 mixture of bone marrow cells from non-transgenic C57BL/6 (Thy1.1) mice and LckOM (Thy1.2) mice (a, c, e), or bone marrow from just C57BL/6 (Thy1.1) animals (b, d, f). Mesenteric node cells were assayed 6 weeks later. a, b, Frequency of cells expressing CD4 and/or CD8 are indicated in each quadrant. c, d, Percentage of CD4⁺CD8⁺ cells that express the Thy1.1 allele. e, f, Percentage of CD4⁺ cells that are Thy1.1⁺.

FIG. 4 Stimulation of T-cell lymphopoiesis in nude mice. CD4 and CD8 expression in mesenteric node cells isolated from: *a*, untreated nude mice; *b*, nude mice 5 weeks after sublethal irradiation (500 cGy); *c*, nude mice 5 weeks after sublethal irradiation plus daily intraperitoneal injections of recombinant human OM (10 µg); *d*, relative per cent of Thy1⁺ mesenteric node cells in the indicated groups of mice ($n = 4$).



Methods

Flow cytometry. Analyses were done with a FACScan flow cytometer (Becton-Dickinson) using Lysis II software. Mouse tissues were isolated and disrupted by standard methods. Cells were stained with fluorescent-conjugated antibodies in the presence of 10% rat serum for 1 h at 4 °C. Antibodies obtained from Pharmingen (San Diego) included phycoerythrin (PE)-conjugated anti-CD4 (RM4-5), fluorescein isothiocyanate (FITC)-conjugated anti-CD8 (53-6.7), FITC-conjugated anti-CD25 (3C7), FITC-conjugated anti-Thy1.2 (30-H12), FITC-conjugated anti-Thy1.1 (HIS51), PE-conjugated anti-heat stable antigen (M1/69), PE-conjugated anti-B220 (RA3-6B2), PE-conjugated anti-CD3 (145-2C11), and the indicated panel of FITC-conjugated anti-Vβ antibodies. Tricolour-conjugated anti-CD4 (YTS 191.1) was obtained from Caltag Laboratories. To enrich for very early thymocyte-like cells (Fig. 1c), we reduced the frequency of T and B cells following incubation with anti-CD8 (AD4; Accurate Chemical & Scientific), anti-CD3 (145-2C11), anti-B220 (RA3-6B2) and rabbit complement (Accurate Chemical & Scientific).

Bone marrow transplants. Donor bone marrow was collected from the femurs and tibias of LckOM transgenic mice, C57BL/6 Thy 1.2 mice (Taconic), and C57BL/6 Thy 1.1 mice (Jackson Laboratory) and depleted of T cells by complement fixation using anti-Thy1.2 antibodies or a combination of antibodies directed against CD8, CD3, and CD4. Cells (5×10^6) were then injected through the tail vein of C57BL/6 nude mice (The Jackson Laboratory) or thymectomized C57BL/6 (Thy1.2) mice that received a lethal dose of irradiation (1000 cGy) from a ¹³⁷Cs source (Shepard, San Francisco).

RT-PCR analysis. Primer sequences and conditions for reverse-transcription polymerase chain reactions have been described⁶.

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1. Roederer, M. *Nature Med.* **1**, 621–622 (1995).
2. Mackall, C. L. et al. *N. Engl. J. Med.* **332**, 143–149 (1995).
3. Franceschi, C., Monti, D., Sansoni, P. & Cossarizza, A. *Immunol. Today* **16**, 12–16 (1995).
4. Malik, N. et al. *Mol. Cell. Biol.* **15**, 2349–2358 (1995).
5. Bruce, A. G., Linsley, P. S. & Rose, T. M. *Progr. Growth Factor Res.* **4**, 157–170 (1992).
6. Yoshimura, A. et al. *EMBO J.* **15**, 1055–1063 (1996).
7. Kisielow, P. & von Boehmer, H. *Adv. Immunol.* **58**, 87–209 (1995).
8. Saint-Ruf, C. et al. *Science* **266**, 1208–1212 (1994).
9. Li, Y. et al. *J. Immunol.* **153**, 421–428 (1994).
10. Gearing, D. P. et al. *Science* **255**, 1434–1437 (1992).
11. Bernad, A. et al. *Immunity* **1**, 725–731 (1994).
12. Escary, J. L. et al. *Nature* **363**, 361–364 (1993).
13. Shen, M. M. et al. *EMBO J.* **13**, 1375–1385 (1994).
14. Yoshida, K. et al. *Proc. Natl Acad. Sci. USA* **93**, 407–411 (1996).
15. Wallace, P. M. et al. *Ann. NY Acad. Sci.* **762**, 42–54 (1995).
16. Rocha, B., Guy-Grand, D. & Vassalli, P. *Curr. Opin. Immunol.* **7**, 235–242 (1995).

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Alternative pathways for the selection of antigen-specific peripheral T cells

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In the thymus, maturing lymphocytes receive activation signals mediated by the T-cell antigen receptor (TCR) that either promote clonal survival (positive selection) or induce apoptosis (negative selection). This balance between life and death is mirrored by the sensitivity of cortical thymocytes to apoptotic death induced by antibodies against the CD3 component of the TCR signal-transduction complex, bacterial superantigens that bind to the TCR β-chain, and corticosteroids^{1–3}. In contrast, mature peripheral T cells are positively activated by anti-CD3 antibody or superantigens and are resistant to steroid-induced death^{4,5}. Here we show that in splenic germinal centres, T cells regain thymocyte-like sensitivity to TCR- and steroid-induced apoptosis and undergo antigen-driven positive and negative selection. T-cell responses elsewhere in the spleen are unaccompanied by programmed cell death. Our observations define a new differentiation pathway for peripheral T cells and suggest that

germinal centres induce a lymphocyte phenotype necessary for the maintenance of self-tolerance.

The majority of T lymphocytes that respond to pigeon cytochrome *c* (PCC) in B10.A mice bear TCRs encoded by the *Vα11* and *Vβ3* gene segments^{6,7} and can be identified in histological sections by immunolabelling and genetic analysis^{8–11}. Average densities for *Vα11*⁺*Vβ3*⁺ T cells in the periarteriolar lymphoid sheath (PALS) or lymphoid follicles of unimmunized B10.A mice are low, about 2 cells and 0.2 cells per $10^4 \mu\text{m}^2$, respectively (Fig. 1a). In the PALS, this density corresponds to 0.3–0.5% of CD4⁺ lymphocytes. Immunization with a haptenated form of pigeon cytochrome *c* (NP-PCC)^{9–11}, but not with other protein conjugates, induces a rapid increase in the numbers of splenic *Vα11*⁺*Vβ3*⁺ lymphocytes within two distinct histological compartments. Initially, clonal expansion is most evident in the PALS, where a 10-fold increase in cell number is present by day 5 of the response. Clonal growth in the PALS is associated with extensive T-cell proliferation (Fig. 1a). After day 5, the number of antigen-specific T cells in the PALS declines to about half of this peak value and continues at this level until day 16 postimmunization (Fig. 1a). The precipitous loss of *Vα11*⁺*Vβ3*⁺ T cells from the PALS is not accompanied by increased apoptotic cell death, as shown by the TUNEL (for TdT-mediated dUTP nick end-labelling) assay^{12,13}.

In contrast to the rapid T-cell response in PALS, NP-PCC induces a steady and more gradual accumulation of *Vα11*⁺*Vβ3*⁺ T cells in lymphoid follicles (Fig. 1a). Nonetheless, by day 16 of the response, PCC-specific follicular T cells are 25 times the number in unimmunized controls, achieving densities comparable to that in the PALS. Before day 5, the increase of *Vα11*⁺*Vβ3*⁺ T cells in the splenic follicles is in part due to immigration^{11,14}; far fewer follicular T cells were labelled by a 2-hour pulse of bromodeoxy-

uridine (BrdU) than in the PALS at this time. However, intense follicular T-cell proliferation is present by day 12 (Fig. 1a), virtually all of it within germinal centres (GCs).

In addition to the dominant use of the *V α 11* and *V β 3* gene segments, selected PCC-specific TCR of B10.A mice have other features, especially CDR3 loop motifs that are believed to be critical for avid recognition of peptide associated with major histocompatibility complex (MHC) molecules¹⁵⁻¹⁷. Typically, these *V(D)J* rearrangements include 8-9-residue α -chain loops containing a glutamate at position 90 and a 9-residue CDR3 β with asparagine at position 100 (refs 15, 16). PCC activates an initially diverse population of *V α 11*⁺*V β 3*⁺ T cells and the subsequent appearance of canonical CDR3 motifs can be used to follow antigen-driven selection¹⁷. We dissected small cohorts of labelled T cells (~20) from histological sections and amplified the *V α 11*–*J α 84* and *V β 3*–*J β 1.2* DNA rearrangements they contained in a multiplex polymerase chain reaction (PCR)¹¹. In the recovered α - and β -chain rearrangements, CDR3 loops quickly converged on sizes appropriate for recognition of PCC peptide (Fig. 1b), followed by the emergence of loop sequences present in PCC-specific T-cell lines and hybridomas (Fig. 1c). Evidence for TCR selection was even more compelling when follicular and GC T cells were considered separately. For example, canonical CDR3 loops were present in 66 and 62%, respectively, of *V α 11*–*J α 84* and *V β 3*–*J β 1.2* rearrangements from GCs on day 12 postimmunization (data not shown).

Figure 1 confirms the maturation of the anti-PCC T-cell response¹⁷; at the peak of antigen-induced clonal expansion in the PALS (day 5; Fig. 1a), only a minority of recovered TCR α - and β -chain rearrangements encoded canonical CDR3 loops, but by two weeks postimmunization, these characteristic TCR rearrangements were in the majority (Fig. 1b, c). As in B-cell responses^{18,19}, clonal dominance among T cells reacting to PCC appears to be achieved by competition and selection^{11,17}.

Competition and selection requires losers as well as winners. For GC B cells, the consequence of losing in antigen-driven selection is thought to be apoptosis²⁰. Could the proliferating, selected populations of GC T cells also be subjected to selective death? A significant fraction, $17 \pm 2\%$ (mean $\pm$ s.e.m.; range =

TABLE 1 Selective apoptosis in germinal centre T cells

		PCR reaction	
		<i>Vα11</i> – <i>Jα84</i>	<i>Vβ3</i> – <i>Jβ1.2</i>
TUNEL ⁺ (<i>n</i> = 48)	<i>n</i> = 28 (58%)	–	–
	<i>n</i> = 16 (33%)	+	–
	<i>n</i> = 4 (8%)	–	+
	<i>n</i> = 0 (0%)	+	+
TUNEL [–] (<i>n</i> = 28)	<i>n</i> = 16 (57%)	–	–
	<i>n</i> = 4 (14%)	+	–
	<i>n</i> = 1 (4%)	–	+
	<i>n</i> = 7 (25%)	+	+

Single TUNEL[–] and TUNEL[–] CD4⁺ cells were dissected from GCs and the *V α 11*–*J α 84* and *V β 3*–*J β 1.2* rearrangements they contained amplified in a multiplex PCR¹¹. As a control, identical amplifications were performed on 66 single, CD4⁺ GC T cells from B10.A mice immunized with NP-CGG. The results of this control: 54 (82%) –/–; 12 (18%) +/–; 0 (0%) –/+; 0 (0%) +/+. None of these *V α 11* rearrangements contained a canonical CDR3 loop sequence.

2–51%) of GC T cells express high levels of a zinc-finger transcription factor, Nur77, which is linked to TCR-mediated apoptosis in immature thymocytes and T-cell hybridomas (Fig. 2)^{21,22}. Most ($\geq 85\%$) of these Nur77⁺ cells also contain the fragmented DNA characteristic of apoptosis, as demonstrated by the TUNEL assay (not shown). This apoptosis is independent of the Fas (APO-1, CD95) death trigger and its ligand; normal numbers of TUNEL⁺ and Nur77⁺ T cells are present in the GCs of immunized *lpr* (Fas^{null}) and *gld* (Fas-ligand^{null}) mice (not shown). In contrast, very few T cells in the PALS express detectable levels of Nur77 (Fig. 2) or are TUNEL⁺ (Fig. 3a). This finding is consistent, even during the proliferative phase of the extrafollicular T-cell response (not shown).

Programmed cell death can be induced in immature thymocytes by activating agents, including crosslinking antibodies specific for the TCR/CD3 complex, bacterial superantigens such as staphylo-

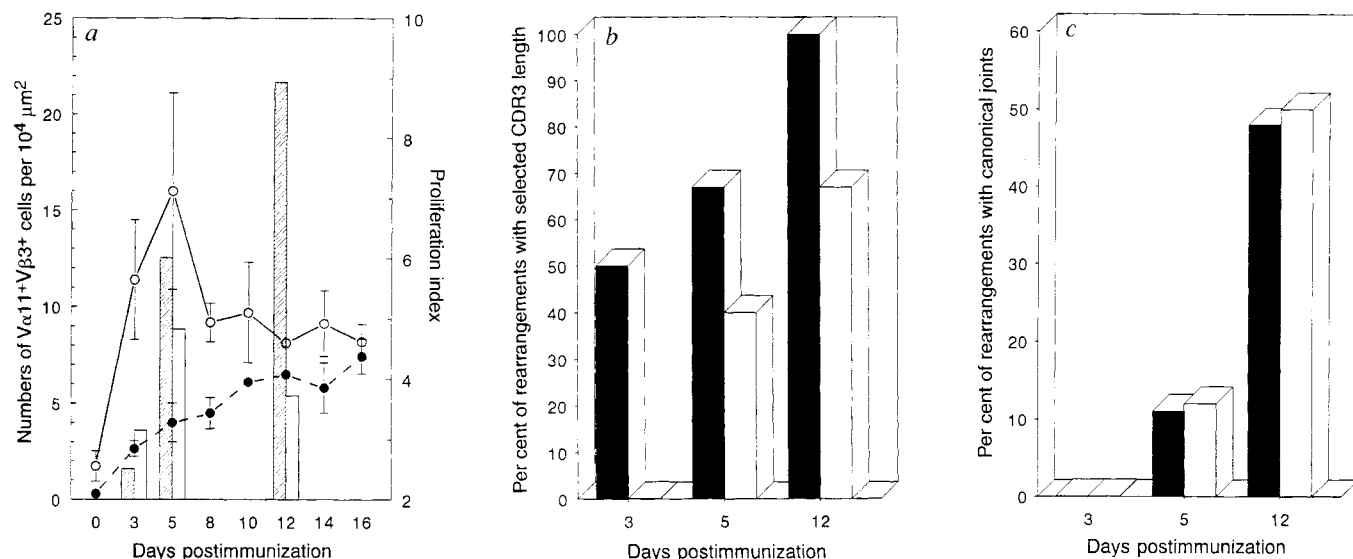


FIG. 1 Kinetics of the expansion and selection of PCC-specific T cells after immunization with NP-PCC. a, Kinetics of the clonal expansion of *V α 11*–*V β 3*⁺ T cells and proliferation index of T cells. The numbers of *V α 11*–*V β 3*⁺ T cells per unit area ($10^4 \mu\text{m}^2$) of PALS (circles) or follicle (filled circles), and the proliferation index in PALS (white bars) or follicle (shaded bars) are indicated. b, selection of rearrangements with a certain CDR3 length. The proportions of *v α 11* rearrangements with a CDR3 of 8 or 9

amino acids (black bars) or *V β 3* rearrangements with a CDR3 of 9 amino acids (white bars) are shown. c, Selection of canonical CDR3 sequences. The percentage of characteristic junctional sequences in all *V α 11*–*J α 84* (black bars) or *V β 3*–*J β 1.2* (white bars) joints is shown. These data combine samples from both PALS and follicular T-cell populations in ratios that approximate the histological distribution of *V α 11*–*V β 3*⁺ T cells.

coccal enterotoxin B (SEB) and glucocorticoids. We examined the sensitivity of GC T cells to these agents. When mice were injected at day 12 postimmunization with doses of a monoclonal anti-CD3 antibody (Fig. 3b), SEB (Fig. 3c) or dexamethasone (Fig. 3d), which is toxic for immature thymocytes, there was a 5–10-fold increase of TUNEL⁺ T cells in GCs (Fig. 3) as well as among cortical thymocytes (not shown) 8 hours after treatment. In contrast, T cells in the PALS were unaffected by these reagents (Fig. 3b–d), and anti-CD3 antibody administered at day 5 of the response did not induce apoptosis in the PALS (not shown). Anti-CD3 antibody also induced massive T-cell death in the GCs and thymuses of *lpr* and *gld* mice (results not shown).

To determine whether programmed cell death acts selectively in GCs, we co-amplified *V α 11-J α 84* and *V β 3-J β 1.2* rearrangements from 48 apoptotic (TUNEL⁺) and 28 live (TUNEL[−]) CD4⁺ GC T cells recovered 12 days after immunization by single-cell microdissection. The results shown in Table 1 indicate that TCR somatic genotypes of these populations are distinct. First, no multiplex amplification of TCR rearrangements from apoptotic T cells yielded both *V α 11* and *V β 3* products, despite a comparable PCR efficiency (42 and 43% respectively) in TUNEL⁺ and TUNEL[−] cells (Table 1). In contrast, 25% (7/28) of live GC T cells gave PCR reactions consistent with the presence of a *V α 11*⁺*V β 3*⁺ TCR. Second, CDR3 loop sequences identical to those of PCC-specific T-cell lines were more prevalent in live cells: 93% of *V α 11* rearrangements recovered from the single TUNEL[−] GC T cells contained canonical CDR3 α -loops compared with 56% in TUNEL⁺ cells. Third, more than half (56%) of the *V α 11-J α 84* fragments recovered from TUNEL⁺ cells contained point mutations, whereas only 9% of the *V α 11-J α 84* rearrangements from TUNEL[−] cells carried misincorporations. Most (8/9) mutations observed in TUNEL⁺ cells were in productive *V α 11* rearrangements, but only five encoded amino-acid replacements. Thus, we can not determine if these misincorporations are a cause or a consequence of apoptosis. However, no *V β 3* rearrangement, even those from TUNEL⁺ cells, contained mutations.

Biased apoptosis in GC T cells is consistent with competitive selection, but could also reflect the death of partially activated, nonspecific lymphocytes. To ensure that antigen-specific GC T cells acquire sensitivity to apoptotic signals, we counted *V α 11*⁺*V β 3*⁺ cells in GCs before and after injection of SEB or dexamethasone (Fig. 4). Within 8 h, these agents kill ~90% of GC T cells bearing *V α 11*⁺*V β 3*⁺ TCR. Together these findings indicate that death comes more often to GC T cells expressing TCRs that do not recognize the PCC antigen appropriately to sustain a competitive growth rate; interestingly, this may preclude the outgrowth of both high- and low-avidity clones²³.

In contrast to the antigen-driven T-cell response in the PALS, apoptosis blunts the expansion of proliferating T cells in GCs. Here, enrichment of antigen-specific T cells is clearly driven by both positive and negative selection. Indeed, a distinctive feature of GC T cells is that they, like immature thymocytes, are prone to Fas-independent apoptosis. Significantly, neither GC T cells nor immature thymocytes express the anti-apoptotic protein Bcl-2, whereas T cells in the PALS express Bcl-2 and are resistant to apoptosis^{11,24}. The GC is a site where both B and T lymphocytes reacquire sensitivity to the selective processes common in primary lymphoid tissues²⁵. This sensitivity may ensure the maintenance of self-tolerance within a microenvironment that promotes the diversification of antigen-receptor genes.

Note added in proof: Phasic proliferation of PCC-specific T Cells in PALS and GCs was recently also demonstrated by A. Gulbranson-Judge and I. C. M. MacLennan²⁹. □

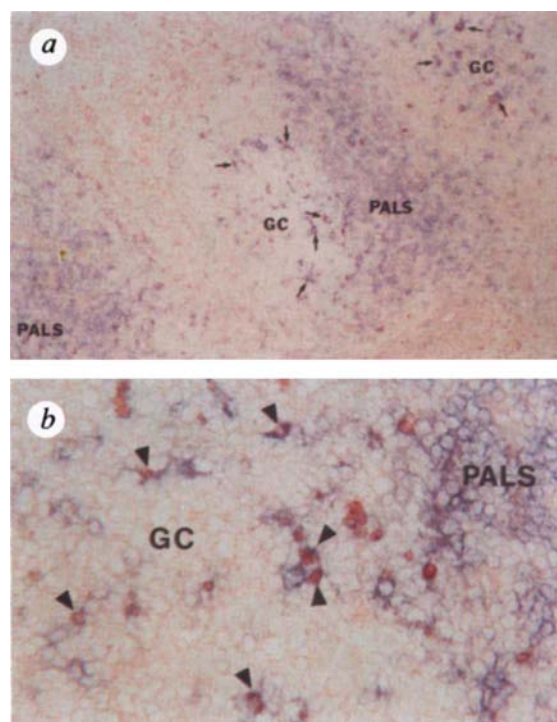


FIG. 2 Nur77 expression in GC T cells. A spleen section 12 days postimmunization was stained with anti-CD4 antibody for T cells (blue) and anti-Nur77 antibody for apoptotic cells (red). a, Apoptotic CD4⁺ Nur77[−] T cells are indicated by arrows. PALS and GCs are also indicated; magnification, $\times 40$. b, Same area as in a; magnification, $\times 200$. Arrowheads, CD4⁺ Nur77⁺ T cells.

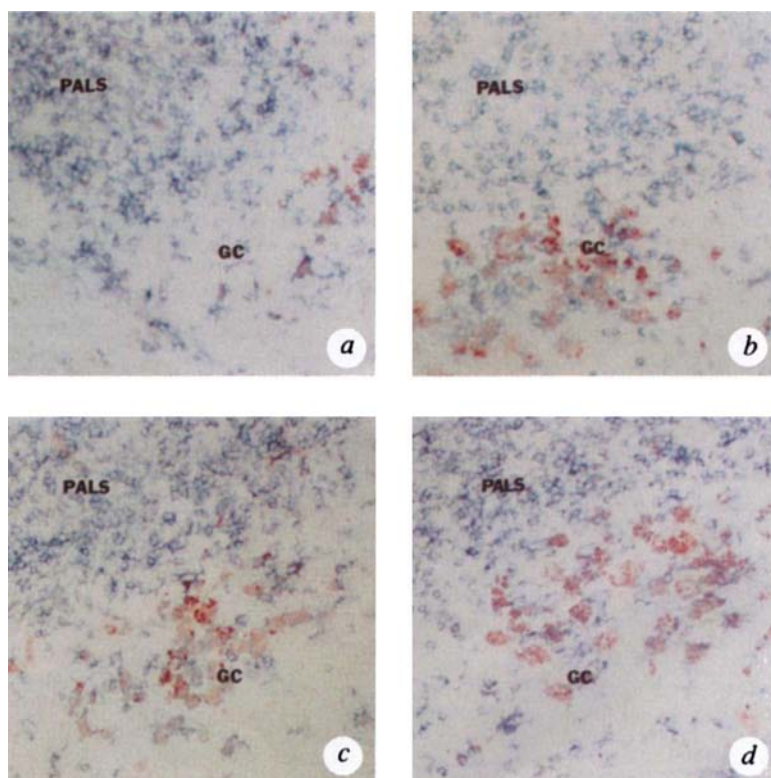


FIG. 3 Induction of apoptosis in GC T cells. Twelve days postimmunization, mice were treated a, with normal hamster immunoglobulin; b, with anti-CD3 antibody; c, with staphylococcal enterotoxin B (SEB); or d, with dexamethasone (DXE). Sections were stained with anti-CD4 antibody for T cells (blue) and TUNEL for apoptotic cells (red). PALS and GCs are indicated.

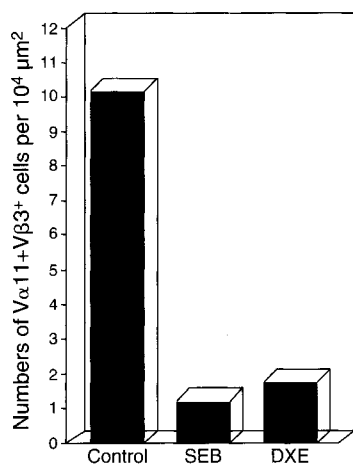


FIG. 4 Loss of Vα11⁺Vβ3⁺ T cells in GCs 8 hours after injection of staphylococcal enterotoxin B (SEB) or dexamethasone (DXE) indicates that PCC-specific GC T cells are fully sensitive to apoptotic signals.

Methods

Mice, immunization and treatments. B10.A, B6.MRL-*lpr* and B6Smn.C3H-*gld* mice (Jackson Laboratory) were immunized intraperitoneally with 100 μg NP-PCC or NP-chicken γ-globulin (NP-CGG) precipitated in alum on day 0 and cohorts of 3–4 mice were killed at times indicated after immunization. In some experiments, at day 12 after immunization, mice were given an intravenous injection of 500 μg normal hamster immunoglobulin (Pierce) or 250 μg anti-CD3 antibody (Pharmingen) or intraperitoneal injections of 500 μg SEB (Sigma) or 500 μg dexamethasone (Sigma); their spleens and thymuses were taken 8 h after treatment.

Immunohistology. Tissue sections were prepared for immunohistology as described^{11,19}. To detect Vα11⁺Vβ3⁺ TCR, sections were stained with antibodies specific for Vα11 TCR²⁶, Vβ3 TCR²⁷ and B220. Procedures for three-colour immunolabelling are described elsewhere¹⁰. The assignment of Vα11⁺Vβ3⁺ T cells to PALS or follicles was defined by B220 staining; PALS are B220⁺ and follicles are B220⁺. For BrdU labelling, mice were given a single intraperitoneal injection of 2 mg BrdU (Sigma) 2 h before killing. After being stained for other cell markers, sections were treated with 1 M HCl for 30 min at 60 °C to expose and partially degrade the DNA. This treatment also serves to terminate the enzymatic reactions that had taken place previously without displacing the precipitates produced by horseradish peroxidase (HRP) and/or alkaline phosphatase (AP). After washing once in water and PBS consecutively, sections were incubated with a mouse monoclonal antibody, BU20a (DAKO A/S, Denmark), specific for BrdU, for 1 h. After washing 3× in PBS/0.1% Tween 20, sections were stained with goat anti-mouse IgG–biotin for 1 h and washed. Finally, sections were incubated with streptavidin–AP for 45 min and washed. BrdU labelling was visualized using fast-red TR/napthol AS/MX (Sigma). The proliferative index is expressed as the number of αβTCR⁺ BrdU⁺ cells per unit area of immunized spleens, divided by the number of αβTCR⁺ BrdU⁺ cells per unit area of unimmunized spleen. To detect local Nur77 expression, sections were stained with biotinylated anti-Nur77, followed by HRP-conjugated streptavidin. To detect apoptotic cells, TUNEL assays were performed using *in situ* apoptosis detection kits purchased from Oncor (Gaithersburg, Maryland).

Microdissection of cells from tissue section, PCR amplification and sequence analysis. T cells from PALS or GC were microdissected from sections using a hydraulic micromanipulator as described^{10,19}. Excised cell matter was incubated with proteinase K overnight at 37 °C and after heat inactivation of the enzyme at 96 °C for 10 min, the lysate was subjected to two rounds of amplification using the polymerase chain reaction (PCR). Amplification of template DNA was by *Pfu* polymerase (Stratagene) using nested primers specific for particular V or J exons. The sequences of PCR primers used for amplification of Vα11–Jα84 and Vβ3–Jβ1.2 rearrangements and PCR conditions are given elsewhere¹⁰. For amplification of DNA from a single cell, the first round of amplification was carried out in 50 μl containing 50 ng 5S rRNA as described¹⁰. The PCR programme consisted of one cycle at 95 °C for 2 min, 55 °C for 4 min, 72 °C for 2 min, followed by 38 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, finally by 1 cycle of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 7 min. 2-μl aliquots of the first-round reaction were reamplified for an additional 40 cycles. Amplified DNA was cloned and sequenced as described^{10,19}. The length of a CDR3 loop was defined as beginning 2 codons upstream of the conserved GYG triplet motif (where G is Gly and Y is any amino acid) in the J region and ending 2 codons downstream of the nearest conserved

V-region cysteine²⁸. Canonical junctions are those Vα11 or Vβ3 CDR3 sequences found in T-cell lines and hybridomas specific for PCC^{15,16}.

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- Smith, C. A., Williams, G. T., Kingston, R., Jenkinson, E. J. & Owen, J. J. T. *Nature* **337**, 181–184 (1989).
- Mercep, M., Weissman, A. M., Frank, S. J., Klausner, R. D. & Ashwell, J. D. *Science* **246**, 1162–1165 (1989).
- Wyllie, A. H. *Nature* **284**, 555–556 (1989).
- Rothenberg, E. *Adv. Immunol.* **51**, 85–214 (1992).
- Cohen, J. J. & Duke, R. C. *Annu. Rev. Immunol.* **10**, 267–293 (1992).
- Winoto, A. et al. *Nature* **324**, 679–682 (1986).
- Hedrick, S. M. *Adv. Immunol.* **43**, 193–234 (1988).
- Fuller, K. A., Kanagawa, O. & Nahm, M. H. *J. Immunol.* **151**, 4505–4512 (1993).
- Kelsoe, G. & Zheng, B. *Curr. Opin. Immunol.* **5**, 418–422 (1993).
- Zheng, B., Xue, W. & Kelsoe, G. *Nature* **372**, 556–559 (1994).
- Zheng, B., Han, S. & Kelsoe, G. *J. Exp. Med.* **184**, 1083–1091 (1996).
- Gavrieli, Y., Sherman, Y. & Ben-Sasson, S. A. *J. Cell Biol.* **119**, 493–501 (1992).
- Surth, C. D. & Sprent, J. *Nature* **372**, 100–1003 (1994).
- Kearney, E. R., Pape, K. A., Luh, D. Y. & Jenkins, M. C. *Immunity* **1**, 327–336 (1994).
- Fink, P. J., Matis, L. A., McEligott, D. L., Bookman, M. & Hedrick, S. M. *Nature* **321**, 219–226 (1986).
- Hedrick, S. M. et al. *Science* **239**, 1541–1544 (1988).
- McHeyzer-Williams, M. G. & Davis, M. M. *Science* **268**, 106–111 (1995).
- McHeyzer-Williams, M. G., McLean, M. J., Larlor, P. & Nossal, G. J. V. *J. Exp. Med.* **178**, 295–303 (1993).
- Jacob, J., Przylepa, J., Miller, C. & Kelsoe, G. *J. Exp. Med.* **178**, 1293–1307 (1993).
- MacLennan, I. C. M. *Annu. Rev. Immunol.* **12**, 117–139 (1994).
- Woronick, J. D., Calnan, B., Ngo, V. & Winoto, A. *Nature* **367**, 277–281 (1994).
- Liu, Z.-G., Smith, S. W., McLaughlin, K. A., Schwartz, L. M. & Osborne, B. A. *Nature* **367**, 281–284 (1994).
- Viola, A. & Lanzavecchia, A. *Science* **273**, 104–106 (1996).
- Vaux, D. L., Cory, S. & Adams, J. M. *Nature* **335**, 440–442 (1988).
- Kelsoe, G. *Immunity* **4**, 107–111 (1996).
- Jameson, S. C. et al. *J. Immunol.* **147**, 3185–3193 (1991).
- Pullen, A. M., Marrack, P. & Kappler, J. W. *Nature* **335**, 796–801 (1988).
- Rock, E. P., Sibbald, P. R., Davis, M. M. & Chien, Y.-H. *J. Exp. Med.* **179**, 323–328 (1994).
- Gulbranson-Judge, A. & MacLennan, I. C. M. *Eur. J. Immunol.* **26**, 1830–1837 (1996).

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Muscle progenitor cells failing to respond to positional cues adopt non-myogenic fates in *myf-5* null mice

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MICE that have mutations in both myogenic transcription factors Myf-5 and MyoD totally lack skeletal muscle fibres and their precursor myoblasts¹, whereas with either mutation alone, muscle is present^{2,3}. Skeletal muscle in the vertebrate body is derived from epithelial somites that respond to environmental signals to form the dorsal epithelial dermomyotome (dermis, muscle) and ventral mesenchymal sclerotome (axial skeleton, ribs)^{4,5}. The first muscle, the myotome, forms centrally in the somite, when only *myf-5* is programming myogenesis. By targeting the *nlacZ* reporter gene into the *myf-5* locus, we demonstrate that β-galactosidase⁺ muscle progenitor cells are present in the dermomyotome of *myf-5* null embryos, and that they undergo a normal epithelial–mesenchymal transition; however, they migrate aberrantly. Dorsally, they accumulate under the ectoderm and express a non-muscle dermal marker, *Derma-1*. Ventrally, β-galactosidase⁺ cells also fail to localize correctly, express a cartilage marker *scleraxis*, and are subsequently found in ribs. Therefore Myf-5 protein is necessary for cells to respond correctly to positional cues in the embryo and to adopt their myogenic fate. In its absence, muscle progenitors, having activated *myf-5*, remain multipotent and differentiate into other somitic derivatives according to their local environment.

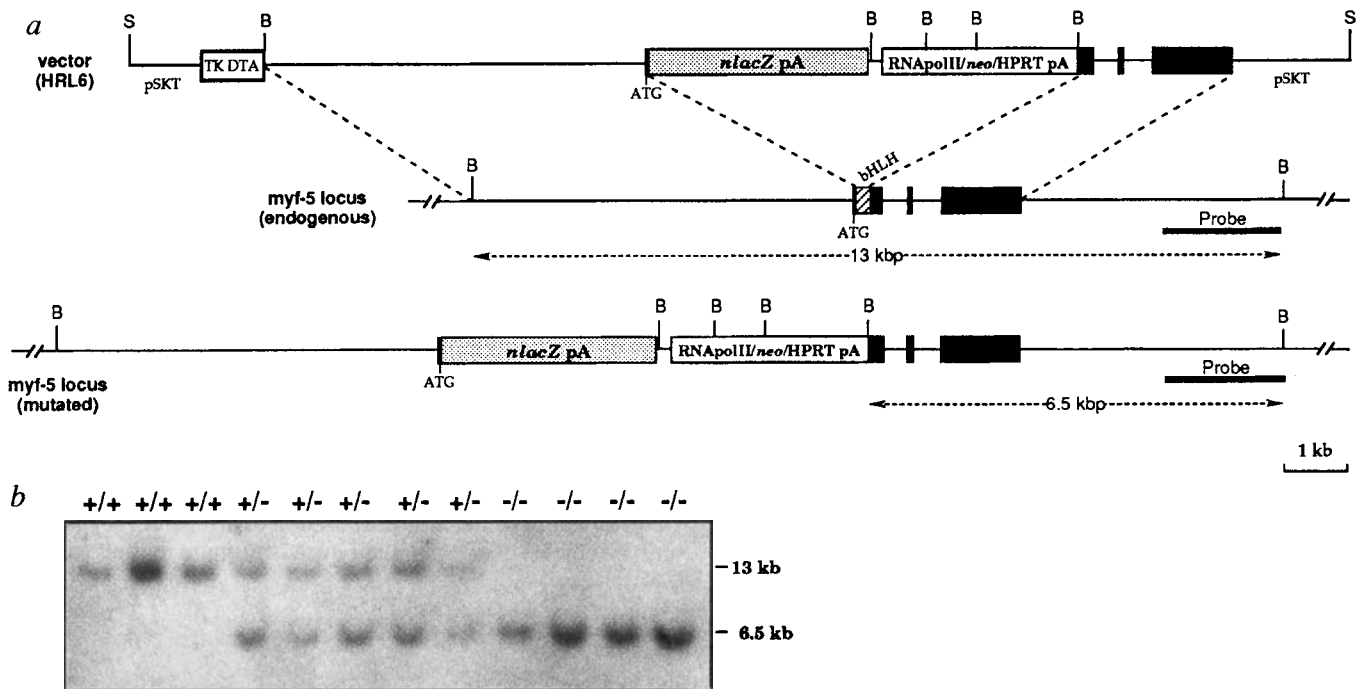


FIG. 1 Gene targeting of the *myf-5* locus with *nlacZ* in ES cells. *a*, The *nlacZ* gene (with SV40 transcription termination and poly(A)⁺ sequences) was followed by a 3.1 kb neomycin gene. The diphtheria toxin A gene driven by a thymidine kinase promoter²⁷ was used for counterselection of non-targeted

events. *b*, Southern analysis of fetal DNA derived from heterozygous crosses. The mutated allele (6.5 kb) is distinguished from the endogenous allele (13 kb) using the probe indicated in *a*. B, *Bam*HI; S, *Sal*I; pSKT (modified Stratagene plasmid).

The delay between the onset of *myf-5* (embryonic day 8 (E8)⁶) and *MyoD* (E10⁷) expression in somites enabled us to examine how *Myf-5* determines muscle progenitor cell fate; we used mice in which *nlacZ* was incorporated into the *myf-5* locus (Fig. 1). At E9.75 in *myf-5*^{a2/+} embryos, β -galactosidase⁺ (β -gal⁺) myotomal cells accumulate under the dermomyotome (Fig. 2*a, b*). Most of these cells are differentiated and express muscle markers including myogenin, but not *MyoD*⁷. In *myf-5*^{a2/-} embryos, muscle progenitors that have activated *myf-5* are indeed present, but display abnormal patterning (Fig. 2*a*). These β -gal⁺ cells do not migrate centrally in the somite but accumulate dorsally and myotome formation is consequently delayed². This perturbation is pronounced by E9.75 in rostral somites where the segmental organization of muscle cells is lost, and at thoracic levels where a ventral accumulation is also observed in the null mutant.

Sections of these embryos demonstrate two features of β -gal⁺ muscle progenitors in homozygous mutants (Fig. 2*c*). First, like those in the wild-type, they delaminate from the dermomyotome, losing their columnar epithelial shape, to become mesenchymal.

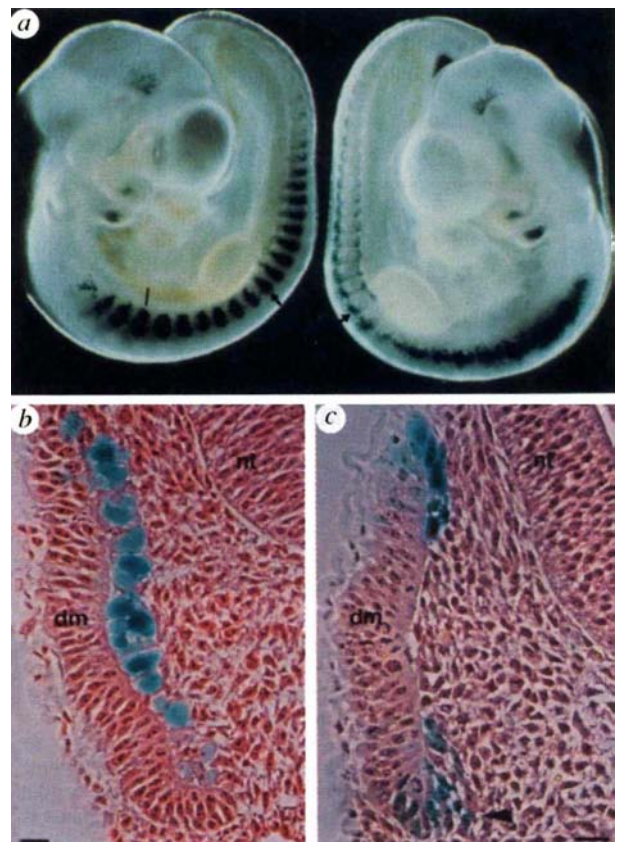


FIG. 2 Abnormal patterning of muscle progenitors in the absence of *myf-5*. *a*, Whole-mount colouration for β -gal activity in *myf-5*^{a2/-} (left) and *myf-5*^{a2/a2/-} (right) E9.75 (31 somites) embryos. Note perturbations in patterning of muscle progenitor cells in the *myf-5*^{a2/-} embryo and the absence of a myotome. Resin sections of heterozygous (*b*) and homozygous (*c*) embryos similar to those shown in *a* at the thoracic level, reveal that β -gal⁺ muscle progenitor cells in the homozygous mutant also leave the dermomyotome, thus undergoing an epithelial-mesenchymal transition (arrowhead in *c* indicating mesenchymal cell in sclerotome compartment), but accumulate along the edges of the dermomyotome (dm) epithelium. β -gal⁺ cells in *b* arise from the dermomyotome to constitute the myotome. The first cervical (C1; 6th) and thoracic (T1; 13th) somites are indicated by a bar and arrow, respectively. nt, Neural tube. Scale bar, 25 μ m.

Second, they migrate aberrantly. Some cells are found ventrally in the sclerotome, whereas other cells become positioned dorsally, adjacent to the ectoderm, in regions that do not normally contain myogenic cells at this stage (see also Fig. 4d, e). This abnormal accumulation of β -gal⁺ cells continues during embryonic development, and remains prominent at caudal levels of E12.5 null embryos. In normal embryos, a basal lamina is produced by myotomal cells which confines them to this somitic compartment⁸. In *myf-5*^{a2-/-} embryos, this basal lamina is missing (Fig. 3). Although this is a consequence of a missing myotome, it probably contributes to the disorganization of the β -gal⁺ progenitors.

The most dramatic disorganization of β -gal⁺ progenitors in *myf-5*^{a2-/-} embryos occurs in the rostral somites by E10.5 (Fig. 4b). These mesenchymal cells are morphologically indistinguishable from their neighbours (Fig. 4d, e). Furthermore, no myogenic markers were detectable in this region at any stage (data not shown). One possibility is that an aberrant location in the embryo results in their death. However, we detected no increase in apoptotic cells in the null mutant (data not shown). Alternatively, the β -gal⁺ cells assume the characteristics of the surrounding tissue. Using *in situ* hybridization with antisense *Dermo-1* we examined whether β -gal⁺ cells from *myf-5*^{a2-/-} embryos had converted to dermal cells. Like the myogenic factors, *Dermo-1* is a basic-helix-loop-helix (bHLH) protein. This gene is expressed in precursor dermal cells from E10.5 but not in skeletal muscle or its precursors⁹. Although *Dermo-1* transcripts are located in cells under the ectoderm and not in the myotome of heterozygous embryos (Fig. 4c), dorsally located cells from homozygous embryos were β -gal⁺ and *Dermo-1*⁺ (Fig. 4d, e). Therefore, based on their location, morphology and expression of *Dermo-1*, our observations suggest that β -gal⁺ cells destined to enter the myogenic lineage will later contribute to dermis in *myf-5*^{a2-/-} embryos.

In thoracic somites, mesenchymal cells in the lateral sclerotome immediately ventral to the myotome condense to form the rib anlagen⁵. In *myf-5*^{a2-/-} embryos, β -gal⁺ muscle progenitors accumulate along the ventrolateral edge of the dermomyotome as mesenchymal cells (Figs 2 and 4). They express *Pax-3*, an early myogenic marker⁴, thus confirming their myogenic progenitor origin (data not shown). In the absence of the myotomal basal lamina they can potentially enter the sclerotome (see Fig. 2c). We investigated whether β -gal⁺ cells entering the rib domain express

the sclerotomal marker *scleraxis*, another bHLH protein that is not found in skeletal muscle¹⁰. At E12.5, whereas intercostal muscles in *myf-5*^{a2+/-} embryos appear as distinct domains between precursor cartilage cells of the ribs (Fig. 5a), in *myf-5*^{a2-/-} embryos, β -gal⁺ muscle progenitors are dispersed among the rib precursors. Many of them express *scleraxis* (Fig. 5b, c). Furthermore, sections of E16.5 fetuses reveal β -gal⁺ cells in the ribs of *myf-5*^{a2-/-} (Fig. 6b) but not *myf-5*^{a2+/-} (Fig. 6a) animals. Their position within the central part of the rib anlagen argues against a simple 'trapping' of intercostal muscle cells along the edges of the rib. Indeed, these β -gal⁺ cells are morphologically indistinguishable from normal cartilage cells and are positive for proteoglycans, as detected histologically by toluidine blue staining, and negative for muscle markers (data not shown). We therefore conclude that these β -gal⁺ cells have entered the cartilage differentiation programme. Other β -gal⁺ cells are present in the disorganized muscle masses, adjacent to the ribs, presumably because of their incorporation into the myogenic programme once *MyoD* is activated². Whether skeletal muscle perturbations or the misincorporation of myogenic progenitors in rib rudiments of *myf-5* mutant mice contribute to their truncated

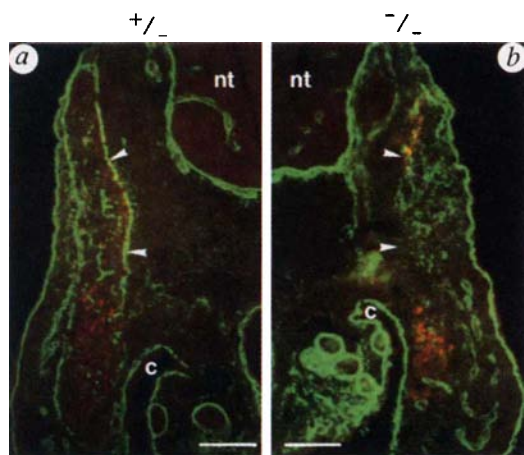


FIG. 3 Myotomal basal lamina is perturbed in *myf-5*^{a2-/-} embryos. Cryostat sections through the thoracic region of E11 heterozygous (a) and homozygous (b) embryos were reacted with anti- β -laminin (polyclonal; green) and anti- β -gal (monoclonal; red) antibodies for detection of basal lamina components and *myf-5* expressing cells, respectively. Note the absence of a basal lamina in the homozygous compared with the heterozygous embryo (arrowheads). Anti-collagen IV yielded similar results (data not shown). nt, Neural tube; c, coelomic cavity delimited by a basal lamina. Scale bar, 100 μ m.

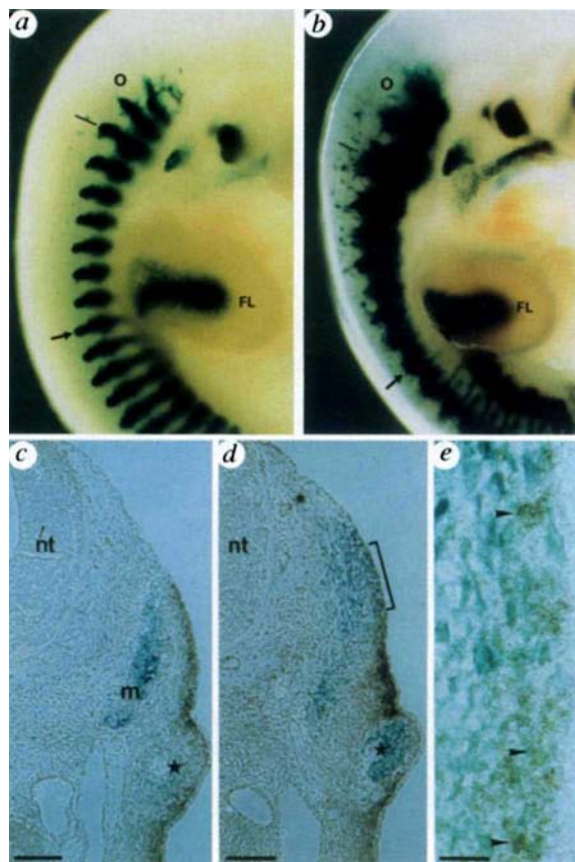


FIG. 4 Progenitor muscle cells located dorsally, exit the somite and express a dermal marker in *myf-5*^{a2-/-} embryos. Although myotomal cells show proper patterning in *myf-5*^{a2-/-} embryos at E10.75 (42 somites) (a), severe perturbations of muscle progenitors in *myf-5*^{a2-/-} embryos (b) were observed in the occipital (o), cervical (C1), and thoracic (T1, arrow) somites. Phase-contrast micrographs of paraffin sections of X-gal-stained heterozygous (c) and homozygous (d) embryos reveal that in the latter, β -gal⁺ cells have left the somite environment and have accumulated under the ectoderm. *In situ* hybridization reveals that *Dermo-1* transcripts (dark grains) normally appear under the ectoderm and remain distinct from myotomal cells (m in c), whereas in the homozygous mutant some cells under the ectoderm (arrowheads) are β -gal⁺ and express *Dermo-1* (e and region indicated by bracket in d). The limit of *Dermo-1* expression at this stage does not extend fully dorsally towards the neural tube to mark more dorsally located β -gal⁺ cells. Asterisks indicate the third branchial arch. nt, Neural tube; FL, forelimb. Scale, 150 μ m for c, d; 25 μ m for e.

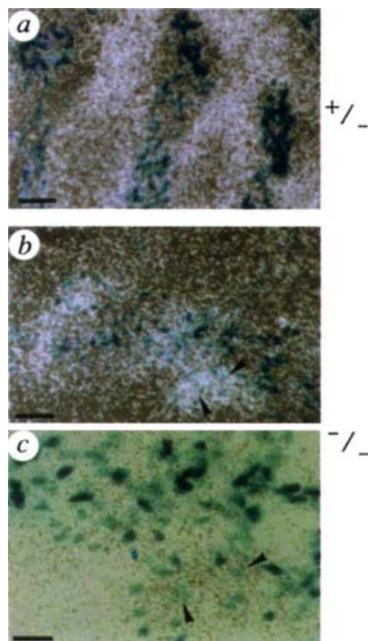


FIG. 5 Ventral β -gal⁺ somitic muscle progenitors in *myf-5* null embryos express a sclerotome marker. Sagittal thoracic cryostat sections of E12.5 *myf-5*^{a2+/-} (a) and *myf-5*^{a2-/-} (b, c) embryos were stained with X-gal and *in situ* hybridized with an antisense scleraxis probe. Although β -gal⁺ cells of intercostal muscles appear distinct from the hybridization signal (white grains in dark field), demarcating condensing cartilage cells of the ribs in heterozygous embryos (a), β -gal⁺ cells in homozygous embryos are dispersed among the condensing cells of the ribs (b). When visualized in the same focal plane (c, magnification of b), the scleraxis signal (black grains in bright field) colocalizes with the X-gal stain (arrowheads). Scale bar, 50 μ m for a, b; 25 μ m for c.

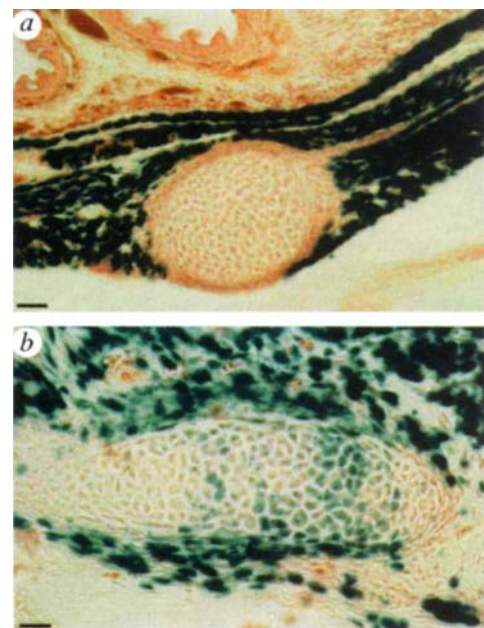


FIG. 6 Muscle progenitor cells in *myf-5* null embryos are found in ribs. Cryostat sections of *myf-5*^{a2+/-} (a) and *myf-5*^{a2-/-} (b) E16.5 fetuses reveal β -gal⁺ cells in the rib of the *myf-5*^{a2-/-} fetus. These β -gal⁺ cells are morphologically distinct from muscle cells, appearing cuboidal in shape like the adjacent cartilage cells. Note also that the muscles in this region are perturbed in the *myf-5*^{a2-/-} fetus. Scale bar, 50 μ m.

rib phenotype, as well as an absence of growth factors normally produced by the early myotome¹¹, is under investigation.

The activation of *myf-5* is a relatively downstream event in myogenic commitment¹². Nevertheless we show that cells that have activated this gene remain multipotent, demonstrating that lineage restriction in mammals, as in *Drosophila*¹³, is a multistep process, where the correct response to positional cues is critical in determining cell fate. The failure of β -gal⁺ myotomal precursors to migrate correctly suggests that Myf-5 is required to activate genes encoding surface proteins, as Pax-3 is presumed to activate the proto-oncogene receptor *c-met* which is required for the migration of Pax-3⁺ myogenic precursors^{14,15}. The fact that cells in *myf-5*^{a2-/-} embryos do leave the dermomyotome is surprising because such an epithelial–mesenchymal transition is often associated with lineage restriction and stabilization of the differentiated state¹⁶. The subsequent acquisition of another somitic fate is apparently due to the mislocation of dispersed muscle progenitor cells. Indeed, in the absence of a ‘community effect’, *Xenopus*¹⁷ and mammalian¹⁸ myogenesis does not proceed. Without appropriate signals, *myf-5* transcription ceases and the β -gal labelling is lost. This occurs earlier in rapidly dividing derm cells than in cartilage. In *Xenopus*¹⁹ and chick^{20–22}, it was not possible to establish whether muscle progenitors, having activated *myf-5* or *MyoD* and turned it off, did switch cell fate in a foreign environment. Using *myf-5*^{a2-/-} mice carrying the *nlacZ* reporter gene, we have now demonstrated this phenomenon. □

Methods

Gene targeting in ES cells and generation of mice. The *myf-5* locus was targeted as described previously²³ with modifications (Fig. 1) involving isolation of the locus (13 kilobase pairs (kbp) *Bam*HI fragment) from an enriched 129sv (D3 ES cell) library. We call this mutation *myf-5*^{a2} to distinguish it from another allele (*myf-5*^{a1}; ref. 23). The *nlacZ* (n, nuclear localization signal) reporter gene

was fused to amino acid 13 in exon 1 creating a null mutation by deleting the bHLH domain (amino acids 14–122). A neomycin cassette (gift from M. Capecchi), containing the RNA polymerase II promoter and 3′ sequences of the HPRT gene (to ensure no transcriptional read through), was introduced after *nlacZ*. Another vector carrying the HSV thymidine kinase gene following the 3′ homology fragment was also used to target HM1 ES cells²⁴ (gift from D. Melton). Neomycin-resistant ES clones (576) were analysed and 6 targeted events were identified by Southern analysis using probes inside and outside the homology fragments. β -galactosidase expression patterns of the targeted cells were verified in chimaeric embryos²⁵. Heterozygous mice obtained from two independent ES cell clones carrying *myf-5*^{a2} were indistinguishable. The mutation was transmitted with mendelian frequencies. Of 682 embryos analysed, 22% were wild type, 51% were heterozygous and 27% were homozygous for the mutation. Heterozygous mice are viable and fertile, whereas mice homozygous for this mutation reproduced the delayed myotome formation, truncated rib phenotype, and perinatal death previously described where the bHLH domain was retained². Sites of *nlacZ* expression in heterozygotes corresponded to those of the endogenous gene, even when unexpected, as in the central nervous system²⁵. The expression pattern of *myf-5*^{a2} was indistinguishable from our previous mutation containing a neomycin cassette with a polyoma enhancer^{12,23,25}. Genotyping details will be described elsewhere (S.T., D.R., G. Cossu and M.B., manuscript in preparation). Stained embryos were embedded and sectioned in paraffin (5–6 μ m), resin (3–4 μ m) or prepared for cryostat (9–12 μ m), and immunohistochemistry was carried out as previously described^{23,25}. Sections were stained with safranin (0.0025%) before examination and photography.

RNA *in situ* hybridization. Embryos at different stages (plug date, E0.5) were fixed, stained with X-gal and embedded in paraffin for *in situ* hybridization as described²⁶. Cryostat sections²⁵ were stained with X-gal (3–5 h), then hybridized. Slides were processed for autoradiography and developed after 10–13 days.

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1. Rudnicki, M. A. et al. *Cell* **75**, 1351–1359 (1993).
2. Braun, T., Rudnicki, M. A., Arnold, H.-H. & Jaenisch, R. *Cell* **71**, 369–382 (1992).
3. Rudnicki, M. A., Braun, T., Hinuma, S. & Jaenisch, R. *Cell* **71**, 383–390 (1992).
4. Cossu, G., Tajbakhsh, S. & Buckingham, M. *Trends Genet.* **12**, 218–223 (1996).
5. Christ, B. & Ordahl, C. P. *Anatomy Embryol.* **191**, 381–396 (1995).
6. Ott, M.-O., Bober, E., Lyons, G., Arnold, H. & Buckingham, M. *Development* **111**, 1097–1107 (1991).
7. Buckingham, M. *Trends Genet.* **8**, 144–149 (1992).
8. Tosney, K. W., Dehnbostel, D. B. & Erickson, C. A. *Dev. Biol.* **163**, 389–406 (1994).
9. Li, L., Cserjesi, P. & Olson, E. N. *Dev. Biol.* **172**, 280–292 (1995).
10. Cserjesi, P. et al. *Development* **121**, 1099–1110 (1995).
11. Grass, S., Arnold, H. H. & Braun, T. *Development* **122**, 141–150 (1996).
12. Tajbakhsh, S. & Buckingham, M. E. *Proc. Natl Acad. Sci. USA* **91**, 747–751 (1994).
13. Siegfried, E. & Perrimon, N. *Bioessays* **16**, 395–404 (1994).

14. Epstein, J. A., Shapiro, D. N., Cheng, J., Lam, P. Y. P. & Maas, R. L. *Proc. Natl Acad. Sci. USA* **93**, 4213–4218 (1996).
15. Yang, X.-M., Vogan, K., Gros, P. & Park, M. *Development* **122**, 2163–2171 (1996).
16. Hay, E. D. *Curr. Opin. Cell Biol.* **5**, 1029–1035 (1993).
17. Gurdon, J. B. *Nature* **336**, 772–774 (1988).
18. Cossu, G., Kelly, R., Di Donna, S., Vivarelli, E. & Buckingham, M. *Proc. Natl Acad. Sci. USA* **92**, 2254–2258 (1995).
19. Godsave, S. F. & Slack, J. M. *Development* **111**, 523–530 (1991).
20. Rong, P. M., Teillet, M. A., Ziller, C. & Le Douarin, N. M. *Development* **115**, 657–672 (1992).
21. Bober, E. et al. *Development* **120**, 3073–3082 (1994).
22. Pownall, M. E., Strunk, K. E. & Emerson, C. P. Jr *Development* **122**, 1475–1488 (1996).
23. Tajbakhsh, S. et al. *Dev. Dyn.* **206**, 291–300 (1996).
24. Magin, T. M., McWhir, J. & Melton, D. W. *Nucleic Acids Res.* **20**, 3795–3796 (1992).
25. Tajbakhsh, S. & Buckingham, M. E. *Development* **121**, 4077–4083 (1995).
26. Tajbakhsh, S. & Houzelstein, D. *Trends Genet.* **11**, 42 (1995).
27. Yagi, T. et al. *Proc. Natl Acad. Sci. USA* **87**, 9918–9922 (1990).

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Interactions of the LIM-domain-binding factor Ldb1 with LIM homeodomain proteins

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THE LIM homeodomain (LIM-HD) proteins, which contain two tandem LIM domains followed by a homeodomain, are critical transcriptional regulators of embryonic development^{1–5}. The LIM domain is a conserved cysteine-rich zinc-binding motif found in LIM-HD and LMO (rhombotin or Ttg) proteins, cytoskeletal components, LIM kinases and other proteins¹. LIM domains are protein–protein interaction motifs¹, can inhibit binding of LIM-HD proteins to DNA^{6,7} and can negatively regulate LIM-HD protein function⁸. How LIM domains exert these regulatory effects is not known. We have now isolated a new LIM-domain-binding factor, Ldb1, on the basis of its ability to interact with the LIM-HD protein Lhx1 (Lim1)⁹. High-affinity binding by Ldb1 requires paired LIM domains and is restricted to the related subgroup of LIM domains found in LIM-HD and LMO proteins. The highly conserved *Xenopus* Ldb protein XLdb1, interacts with Xlim-1, the *Xenopus* orthologue of Lhx1. When injected into *Xenopus* embryos, XLdb1 (or Ldb1) can synergize with Xlim-1 in the formation of partial secondary axes and in activation of the genes encoding gooseoid (*gsc*), chordin, NCAM and XCG7, demonstrating a functional as well as a physical interaction between the two proteins.

To isolate complementary DNAs encoding LIM-binding factors, a protein probe containing the LIM domains of Lhx1 (Fig. 1b, left) was used to screen a mouse embryonic cDNA expression library. Of five positive cDNA clones isolated, four were derived from the same gene and encode a 375-amino-acid (*M_r* 43K) protein, designated Ldb1, for LIM-domain-binding protein 1 (Fig. 1c). No known motifs were found in the sequence, except for a possible nuclear-localization signal at positions 259–264 and 284–286. The fifth cDNA clone encoded a protein that is closely related to Ldb1, named Ldb2, and which will be described else-

where. Ldb1 and Ldb2 show homology to several human expressed-sequence tags of unknown function. *Xenopus* Ldb1, XLdb1, cloned by cross-hybridization, is 98% identical to mouse Ldb1 (Fig. 1c). XLdb1 was also isolated by the two-hybrid system in yeast from a *Xenopus* gastrula-stage library using the Xlim-1 LIM domains as bait (Fig. 1b, right). Of 29 positive clones, 18 contained XLdb1-coding sequences. Thus, XLdb1 can interact with LIM domains *in vivo*.

Specificity of the Ldb1–LIM-domain interaction was assayed by far-western blotting using immobilized Ldb1 protein. Figure 2a shows the induction of Ldb1 protein. Duplicates of this blot were probed with LIM-domain-containing proteins. Figure 2b verifies that the radioactivity of the protein probes is about equal. Full-length Lhx1 protein binds strongly to Ldb1, but Lhx1 mutant proteins 1m, 2m and 3m, which contain point mutations in conserved cysteine residues in LIM-A, LIM-B or in both LIM domains, respectively (Fig. 1a, bottom), bind only very weakly to Ldb1 (Fig. 2c). The same results were obtained using Xlim-1, and the 1m, 2m and 3m mutants of Xlim-1 as probes (data not shown)^{8,10}. This indicates that Ldb1 binds to Lhx1/Xlim-1 in a highly specific manner, and that both intact LIM domains are required for strong binding. Specific binding is also indicated *in vivo*, because wild-type Xlim-1 LIM domains bound XLdb1 in yeast, but 3m mutant LIM domains did not (Fig. 2f).

LIM domains can be grouped into five classes (A–E) by sequence homology^{1,11}. LIM-HD and LMO proteins all have an A-type followed by a B-type LIM domain (AB-type proteins), whereas the domains in LIM kinases (LIMK)¹² are marginally classified as AB types. MLP (muscle LIM protein)¹³ is a protein with two C-type domains; zyxin¹⁴ has two D- and one E-type domain. Ldb1 binding to the various classes of LIM proteins was tested by far-western blotting using full-length protein probes

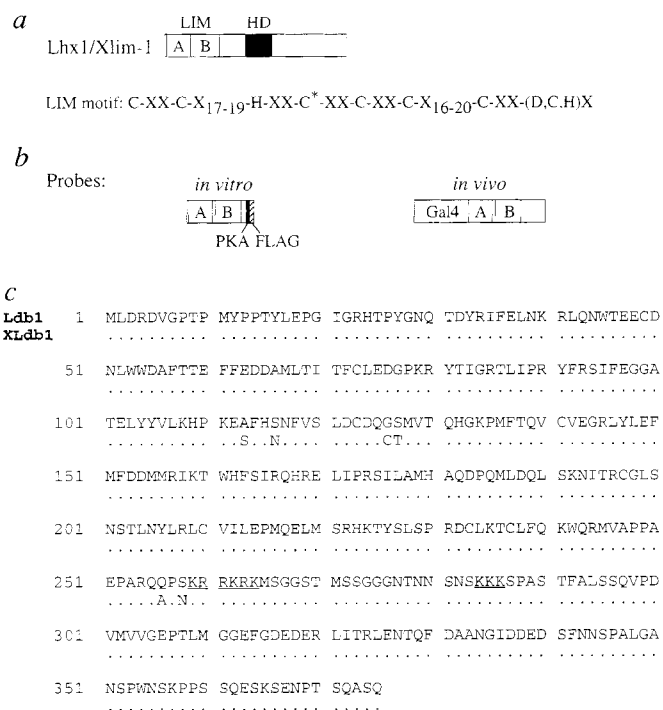


FIG. 1 Ldb1 and XLdb1 cloning. **a**, Structure of Lhx1 (Lim1) and Xlim-1 proteins and the LIM-domain consensus sequence. A and B are the first and second LIM domains, respectively; HD is the homeodomain; the asterisk indicates a cysteine-to-glycine point mutation (see text, and Figs 2 and 4). **b**, LIM-domain probes used for cDNA library screening by direct protein binding (*in vitro*) and by the 2-hybrid system (*in vivo*). PKA, protein kinase A recognition site; Flag, epitope tag; Gal4, DNA-binding domain. **c**, The deduced amino-acid sequences of Ldb1 and XLdb1; dots signify identity. A potential nuclear localization signal is underlined.

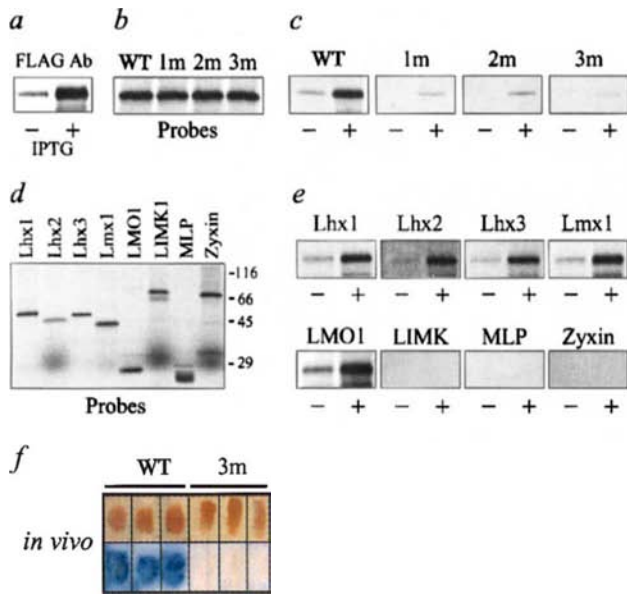


FIG. 2 Specificity of Ldb1 and XLdb1 binding. *a*, Induction of FLAG epitope-tagged Ldb1 protein, as visualized by western blotting with an anti-Flag antibody; (–) and (+), uninduced and IPTG-induced cell extracts, respectively. *b* and *d*, 35 S-labelled protein probes analysed by SDS-PAGE. *c* and *e*, Far-western blots of Ldb1 protein probed with labelled LIM proteins. Weak reactivity in uninduced lanes is due to leakiness in the Lac repressor system. *f*, Binding of Xlim-1 to XLdb1 *in vivo*. Yeast was transformed with plasmids expressing Gal4 activation domain–XLdb1 and either the Gal4 DNA binding domain fused to the Xlim-1 LIM domains wild type (WT) or 3m mutant. Top row, yeast colonies; bottom row, β -galactosidase reporter gene staining.

containing similar amounts of radioactivity (Fig. 2*d*). The LIM-HD proteins Lhx1, Lhx2 (LH-2), Lhx3 (Lim3 or P-Lim) and Lmx1 and the LIM-only protein LMO1 (and LMO2; not shown) all bound strongly to Ldb1 (Fig. 2*e*)^{14–17}. In contrast, no binding was detectable for LIMK1, MLP and zyxin (Fig. 2*e*). Ldb1, when used as a probe in this assay, does not bind to itself (not shown). These results indicate that among the known classes of LIM proteins, Ldb1 binds only to AB types. The HLH proteins Tal1 and Pan1, and the POU factor Pit-1 interact physically or functionally with the AB-type LIM domain proteins LMO2, Lmx1 and Lhx3 (P-Lim), respectively^{16–18}. It will be of interest to see whether Ldb1 can interact with or compete for LIM-domain binding with any of these proteins.

Expression of Ldb1 and XLdb1 was analysed by northern blotting (Fig. 3). Two major Ldb1 messenger RNA transcripts of 2.4 and 3.7 kilobases (kb) were seen in mouse embryos and in multiple tissues in adults (Fig. 3*a*). XLdb1 was expressed as a 3.0-kb mRNA in blastula and gastrula-stage *Xenopus* embryos (Fig. 3*b*). As shown in Fig. 3*c*, in contrast to Xlim-1, XLdb1 was ubiquitously expressed in the early gastrula. Xlim-1 and XLdb1 are co-expressed in the dorsal region of the vegetal hemisphere, which contains the Spemann organizer.

To investigate possible functional interactions between Ldb1/XLdb1 and LIM-HD proteins, we injected mRNA into *Xenopus* embryos (Fig. 4). The 3m LIM-domain mutant of Xlim-1 is known to initiate secondary axis formation with associated neural and muscle differentiation when it is ectopically expressed in the ventral equatorial region of *Xenopus* embryos, whereas wild-type Xlim-1 only has weak activity⁸. This result indicated that LIM domains might act as negative regulatory elements, causing a mutation in the LIM domains to produce an activated form of Xlim-1. We considered that during normal development, Xlim-1 is activated by interaction with Ldb1. Injection of either Xlim-1 or Ldb1 alone had little effect on embryo development; however, co-injection of Ldb1 and Xlim-1 mRNAs synergistically induced

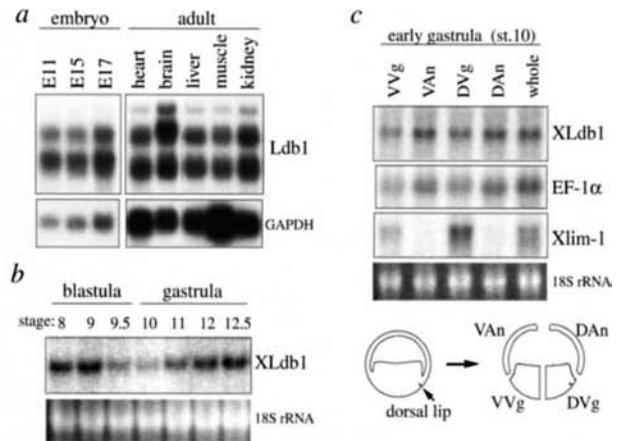


FIG. 3 Northern blot analysis of Ldb1 and XLdb1. *a*, Mouse embryo and adult mRNA. E11, E15, E17, days post-coitum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase hybridization control. *b*, *Xenopus* stage-specific mRNA. *c*, Distribution of mRNAs in *Xenopus* gastrula stage embryos. D, dorsal; V, ventral; An, animal; Vg, vegetal; EF-1 α , elongation factor-1 α hybridization control.

partial secondary axes or a dorsalized phenotype (Fig. 4*a*). Ectopic muscle formation was seen in such embryos either along distinct secondary axes (Fig. 4*b*, bottom left) or as fused right and left somites (Fig. 4*b*, bottom right). The high frequency of ectopic muscle formation in co-injected embryos clearly shows the synergism of their action (Fig. 4*c*), as we also found when XLdb1 mRNA was used in similar experiments (not shown).

The Xlim-1 3m point mutant or the LIM-domain deletion mutant Δ NA (with both LIM domains deleted) can initiate expression of the neural marker NCAM and the cement gland marker XCG7 in animal explants⁸. Co-expression of Xlim-1 with Ldb1 or XLdb1 in animal explants initiated the expression of NCAM and XCG7 (Fig. 4*d*, left), and of the organizer markers goosecoid and chordin (Fig. 4*e*). In contrast, injection of Xlim-1, XLdb1 or Ldb1 alone had little or no effect. Thus, Ldb1/XLdb1 can synergize with Xlim-1 to create ectopic secondary axes and to activate gene expression; these phenotypes are similar to those obtained with the activated Xlim-1 mutants 3m or Δ NA. When XLdb1 is co-injected with 3m or Δ NA, levels of NCAM and XCG7 expression are not further enhanced (Fig. 4*d*, right), indicating that intact LIM domains are required for synergy with XLdb1.

Together with our data indicating that Ldb1/XLdb1 binds specifically to the LIM domains of Lhx1/Xlim-1 *in vitro* and *in vivo* (Fig. 2), these embryo injection experiments suggest that direct binding of Ldb1/XLdb1 to Xlim-1 can activate Xlim-1 function *in vivo*. Consistent with this idea, XLdb1 (Fig. 4*f*), Ldb1 (not shown) and Xlim-1 (ref. 19) proteins localize to the nucleus. Finally we note that XLdb1 and Xlim-1 are naturally co-expressed in the dorsal region of the *Xenopus* gastrula where their interaction could be involved in Spemann organizer activity. Given its *in vitro* binding specificity (Fig. 2*d*, *e*) and broad expression pattern (Fig. 3), Ldb1/XLdb1 may also functionally interact with other LIM-HD proteins and with LMO proteins as well. □

Methods

Library screening. A bacterial Lhx1 expression vector was constructed in pFLAG-CTC (Kodak/IBI) encoding the Lhx1 LIM domains (amino acids 1–128), followed by a phosphorylation site (RRASV) for protein kinase A (PKA), and the Flag (Kodak/IBI) epitope tag. Recombinant Lhx1 protein was partially purified on a Flag M2 antibody-agarose column (Kodak/IBI) and 32 P-labelled with the catalytic subunit of PKA (Sigma) as described²⁰. Labelled protein was purified on a G-50 column in overlay buffer (20 mM HEPES, pH 7.7, 10 mM NaCl, 1 mM DTT, 1 mM EDTA, 1% NP-40, 1% Carnation non-fat dry milk; method modified

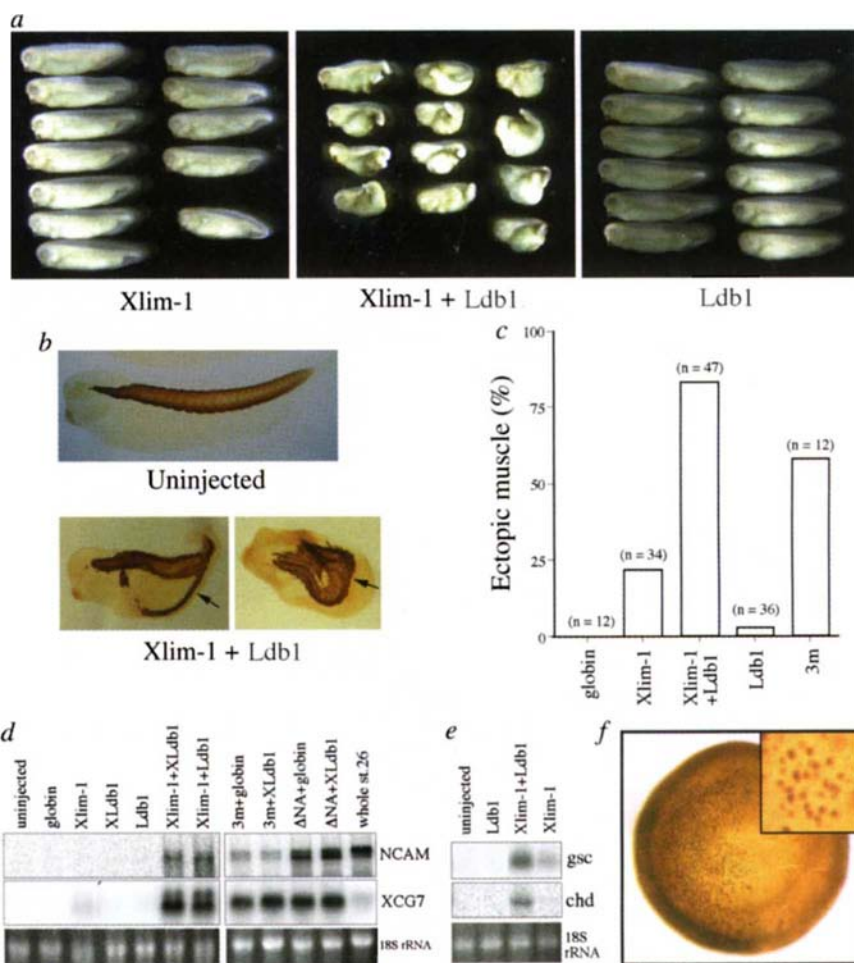


FIG. 4 Synergistic activity of Xlim-1 and Ldb1/XLdb1. **a**, *Xenopus* embryos injected at the 4-cell stage with 0.25 ng of each RNA into the ventral equatorial region. **b**, Whole-mount immunostaining. Xlim-1 + Ldb1-injected embryos showed ectopic muscle formation (arrows) in the ventral region (left), or a dorsalized phenotype (right), with shortened tail and fused somites. **c**, Incidence of ectopic muscle formation. **d** and **e**, Synergistic gene activation by Xlim-1 and Ldb1/XLdb1 in animal explants, assayed by northern blot at the tail-bud stage (**d**) or the gastrula stage (**e**). **f**, Immunostaining at gastrula stage of Flag-tagged XLdb1 protein shows nuclear localization; mRNA was injected into one blastomere at the 2-cell stage.

from ref. 21). For *in vitro* interaction screening, filters containing induced proteins from 0.5×10^6 phage (mouse E11 library, Clontech) were washed 4 times in overlay buffer for 1 h, probed with ^{32}P -labelled LIM domains in the same buffer overnight at 4°C or for 4 h at room temperature, and washed in the same buffer 3 times for 20 min. The two-hybrid screen used a bait construct containing the Xlim-1 LIM domains (amino acids 1–177) in pAS2-1 (Clontech) and a *Xenopus* gastrula-stage cDNA library in pGAD10 (Clontech); 2×10^6 transformants in the yeast strain CG-1945 (Clontech) were screened²². Of 323 His⁺ clones, 29 were His⁺ lacZ⁺, and 18 encoded XLdb1.

Binding assays. A prokaryotic vector expressing Flag-tagged Ldb1 was constructed by replacing Lhx1 coding sequences in the Lhx1 expression plasmid with a product from polymerase chain reaction (PCR) encoding full-length Ldb1. Bacterial extracts were subjected to SDS–PAGE and transferred to nitrocellulose filters. For western blotting, the filter was probed with anti-Flag M2 monoclonal antibody (Kodak/IBI). For far-western blots, LIM proteins were synthesized and ^{35}S -labelled *in vitro* (TNT kit, Promega). Ldb1-containing filters were blocked for at least 1 h in overlay buffer, and probed as described. For binding assays in yeast, the wild-type construct was the bait construct and the 3m mutant contained the two point mutations described in the text. XLdb1 was cloned into the pACT2 Gal4-activation domain plasmid (Clontech). Plasmids were transformed into strain Y190 (Clontech) (ref. 22), and stained for β -galactosidase.

Northern blotting. Mouse embryo and adult-tissue northern blots (Clontech) contained 2 μg per lane poly(A)⁺ mRNA and *Xenopus* blastula and gastrula-stage blots contained 10 μg (Fig. 3b) or 7 μg (Fig. 3c) per lane total RNA. Expression analysis of goosecoid, chordin, NCAM and XCG7 in injected embryos used 7 μg (Fig. 4d) or 4 μg (Fig. 4e) total RNA per lane. RNA extraction and hybridization were done as described⁸.

Xenopus embryo injections and immunostaining. Plasmids for mRNA injections were constructed by PCR amplification of Ldb1 and XLdb1 coding sequences, and cloning into pSP64RI. For immunostaining, the XLdb1 construct was epitope-tagged at the N terminus by insertion of a Flag-encoding oligonucleotide; Flag-Ldb1 was made by PCR cloning Flag-tagged Ldb1 into pSP64RI. Xlim-1 and globin constructs, mRNA synthesis, *Xenopus* embryo injections, immunostaining and animal cap assays have been described⁸.

Muscle was analysed using the 12/101 antibody²³; staining of Flag-tagged proteins used anti-Flag M2 antibody (Kodak/IBI).

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- Dawid, I. B., Toyama, R. & Taira, M. *C. R. Acad. Sci. III* **318**, 295–306 (1995).
- Sanchez-Garcia, I. & Rabbitts, T. H. *Trends Genet.* **10**, 315–320 (1994).
- Shawlot, W. & Behringer, R. R. *Nature* **374**, 425–430 (1995).
- Pfaff, S. L., Mendelsohn, M., Stewart, C. L., Edlund, T. & Jessell, T. M. *Cell* **84**, 309–320 (1996).
- Sheng, H. Z. *et al. Science* **272**, 1004–1007 (1996).
- Xue, D., Tu, Y. & Chalfie, M. *Science* **261**, 1324–1328 (1993).
- Sanchez-Garcia, I., Osada, H., Forster, A. & Rabbitts, T. H. *EMBO J.* **12**, 4243–4250 (1993).
- Taira, M., Otani, H., Saint-Jeannet, J.-P. & Dawid, I. B. *Nature* **372**, 677–679 (1994).
- Fuji, T. *et al. Dev. Dyn.* **199**, 73–83 (1994).
- Taira, M., Jamrich, M., Good, P. J. & Dawid, I. B. *Genes Dev.* **6**, 356–366 (1992).
- Taira, M., Evrard, J. L., Steinmetz, A. & Dawid, I. B. *Trends Genet.* **11**, 431–432 (1995).
- Nunoue, K., Ohashi, K., Okano, I. & Mizuno, K. *Oncogene* **11**, 701–710 (1995).
- Arber, S., Halder, G. & Caroni, P. *Cell* **79**, 221–231 (1994).
- Sadler, I., Crawford, A. W., Michelsen, J. W. & Beckerle, M. C. *J. Cell. Biol.* **119**, 1573–1587 (1992).
- Zhadanov, A. B., Bertuzzi, S., Taira, M., Dawid, I. B. & Westphal, H. *Dev. Dyn.* **202**, 354–364 (1995).
- Geman, M. S., Wang, J., Chadwick, R. B. & Rutter, W. J. *Genes Dev.* **6**, 2165–2176 (1992).
- Bach, I. *et al. Proc. Natl Acad. Sci. USA* **92**, 2720–2724 (1995).
- Wadman, I. *et al. EMBO J.* **13**, 4831–4839 (1994).
- Karavanov, A. A., Saint-Jeannet, J.-P., Karavanova, I., Taira, M. & Dawid, I. B. *Int. J. Dev. Biol.* **40**, 453–461 (1996).
- Blanan, M. A. & Rutter, W. J. *Science* **256**, 1014–1018 (1992).
- Crawford, A. W., Michelsen, J. W. & Beckerle, M. C. *J. Cell Biol.* **116**, 1381–1393 (1992).
- Harper, J. W., Adams, G. R., Wei, N., Keyomarsi, K. & Elledge, S. J. *Cell* **75**, 805–816 (1993).
- Kintner, C. R. & Brockes, J. P. *Nature* **308**, 67–69 (1984).

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Three distinct signalling responses by murine fibroblasts to genotoxic stress

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GENOTOXIC stress triggers signalling pathways that mediate either the protection or killing of affected cells. Whereas induction of p53 involves events in the cell nucleus¹, the activation of transcription factors AP-1 and NF- κ B by ultraviolet radiation is mediated through membrane-associated signalling proteins, ruling out a nuclear signal²⁻⁶. An early event in AP-1 induction by ultraviolet radiation is activation of Jun kinases (JNKs)^{3,7}, which mediate the induction of the immediate-early genes *c-jun* and *c-fos*⁷⁻¹³. The JNKs have also been proposed to mediate the apoptotic response to genotoxins¹⁴. The non-receptor tyrosine kinase c-Abl is also activated by genotoxic stress^{15,16}. To understand the relationship between these events, we compared the activation of p53, JNK and c-Abl by several DNA-damaging agents in murine fibroblasts. We found that whereas p53 was induced by every genotoxic stimulus tested, c-Abl was activated by most stimuli except ultraviolet irradiation and JNK was strongly stimulated only by ultraviolet light and the alkylating agent methyl methanesulphonate. Activation of JNK by this alkylating agent was normal in c-Abl-null cells but was reduced in c-Src-null cells. Unlike p53 induction, c-Abl activation occurs in the S phase of the cell cycle and does not affect cell proliferation. These findings show that signals generated by genotoxins are transduced by multiple, independent pathways. Only p53 appears to be a universal sensor of genotoxic stress.

Phosphorylation of the C-terminal repeat domain (CTD) of the large subunit of RNA polymerase II, a substrate for c-Abl^{17,18}, was used to measure c-Abl activity by an immune-complex kinase assay (Fig. 1). Kinase activity was dependent on c-Abl expression, as Abl antibody did not precipitate CTD kinase from c-Abl-null cells (3T3-Abl^{-/-}) (Fig. 1b, c, e, lanes 4-6). Reintroduction of the c-abl gene into *abl*^{-/-} cells (3T3-Abl⁺) restored CTD-kinase

activity (Fig. 1a, lanes 7, 8; Fig. 1b, c, e, lanes 7-9). As previously reported¹⁵, ionizing radiation and cisplatin (CDDP), which both damage DNA, stimulate c-Abl activity. Similar stimulation was seen in response to methyl methanesulphonate (MMS) treatment, whereas mitomycin C was less effective. Short-wavelength ultraviolet radiation (UV-C), a potent JNK activator^{3,7,8},

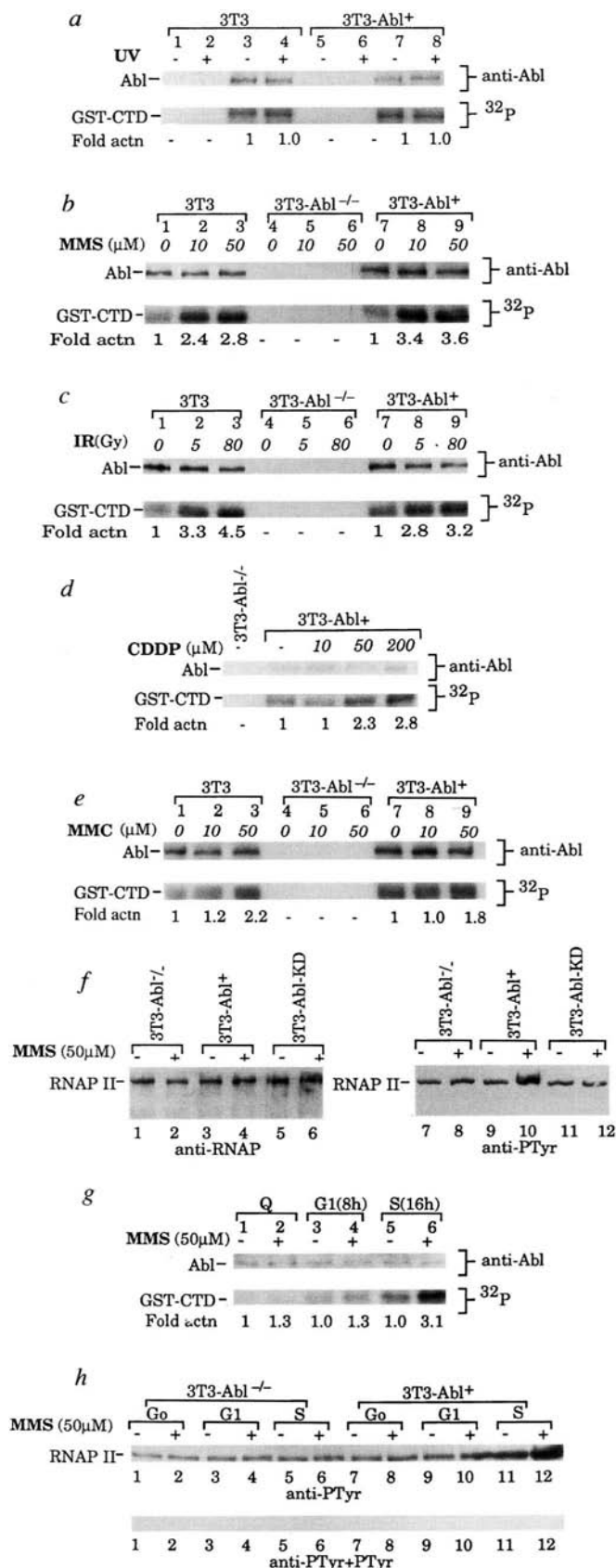


FIG. 1 Effect of genotoxic agents on c-Abl tyrosine kinase activity. a, Cells were irradiated with UV-C (40 J m⁻²) and collected 1 h later. c-Abl kinase was assayed by immune-complex kinase assays with GST-CTD as a substrate²⁴. Lanes 1, 2, 5, 6: immunoprecipitation with normal rabbit serum; lanes 3, 4, 7, 8: immunoprecipitation with c-Abl antiserum. Immune complexes were examined for their c-Abl content by immunoblotting with c-Abl antibody (top panel) or used for GST-CTD phosphorylation (bottom panel). b-e, Cells were treated with MMS, CDDP, or mitomycin C (MMC) for 60 min and collected, or exposed to γ -irradiation (IR) and collected 1 h later. f, Stimulation of tyrosine phosphorylation of RNAP II large subunit by MMS *in vivo*. Abl^{-/-} cells, Abl⁺ cells or Abl^{-/-} cells expressing catalytically inactive Abl (3T3-Abl-KD) were incubated with either normal medium (-) or in the presence of 50 μ M MMS (+) for 1 h and lysed. The large subunit of RNAP II was immunoprecipitated and its phosphotyrosine content determined by immunoblotting. g, Stimulation of c-Abl kinase by MMS in S phase. NIH3T3 cells were synchronized in G0 (Q), G1 or S phase before treatment with 50 μ M MMS. c-Abl content and activity were determined as described. h, *In vivo* stimulation of tyrosine phosphorylation of RNAP II large subunit in S phase by MMS. Abl^{-/-} or Abl⁺ cells synchronized at the indicated cell-cycle phases were incubated in the absence or presence of MMS for 1 h and RNAP II large-subunit tyrosine-phosphorylation examined. Fold actn, fold activation.

did not stimulate c-Abl activity (Fig. 1a). Stimuli such as MMS augmented tyrosine-phosphorylation of the large subunit of RNA polymerase II in living cells, but only in those expressing a functional c-Abl kinase (Fig. 1f).

The nuclear c-Abl tyrosine kinase is regulated by the cell cycle^{19,20}. In resting cells or cells in early G1 phase, binding of the retinoblastoma protein Rb to c-Abl tyrosine-kinase domain inhibits kinase activity. As Rb becomes phosphorylated during progression through the G1/S phases of the cell cycle, c-Abl is released and activated^{19,20}. After cells enter S phase, an increase in basal c-Abl activity is seen (Fig. 1g; compare lanes 1, 3 and 5). We therefore tested whether c-Abl activation by genotoxic agents depends on the cell cycle. 3T3 cells made quiescent by serum deprivation were restimulated to enter the cell cycle and treated with MMS at three time points (Fig. 1g). MMS treatment stimulated c-Abl activity only 16 hours after serum addition. Fluorescence-activated cell sorting (FACS) analysis indicated that at this point the cells had entered S phase (data not shown). Correspondingly, MMS stimulated tyrosine-phosphorylation of RNA polymerase II only in S phase cells expressing a functional c-Abl (Fig. 1h). These results strongly indicate that the large subunit of RNA polymerase II is a bona fide c-Abl substrate.

As JNK may also be a universal effector of genotoxic stress^{15,16},

we examined the activation of endogenous JNK as well as of a transfected haemagglutinin (HA)-tagged JNK (refs 8, 21) by the agents already tested. The extent of endogenous Jnk1 activation by UV-C was identical in NIH3T3, 3T3-Abl^{-/-} and 3T3-Abl^{+/+} cells (Fig. 2a; data not shown for NIH3T3 cells). Similar results were obtained with in-gel kinase assays, which examine both Jnk1 and Jnk2 activities²¹ (data not shown). As found previously¹⁰, Jnk1 activity was strongly stimulated by MMS, a response that was independent of c-Abl expression (Fig. 2a). Jnk1 was moderately stimulated by mitomycin C or CDDP at high doses (Fig. 2b, c) and was not activated by ionizing radiation in established 3T3 cells (Fig. 2d). Similar results were obtained when transiently expressed HA-Jnk1 was analysed for its response to the various treatments (data not shown). Doses of mitomycin C, CDDP or ionizing radiation that had little effect on JNK activity induced maximal p53 accumulation (Fig. 2b–d), suggesting that substantial DNA damage had been induced without JNK activation. We also examined the response of JNK to ionizing radiation in primary fibroblasts isolated from *c-abl*^{+/+} or *c-abl*^{-/-} mouse embryos and found that JNK activity was moderately stimulated, but only at a very high dose of 200 Gy. No difference was seen between the primary *c-abl*^{+/+} and *c-abl*^{-/-} embryo fibroblasts (Fig. 2e). We previously reported that v-Abl activates JNK (ref. 22), but

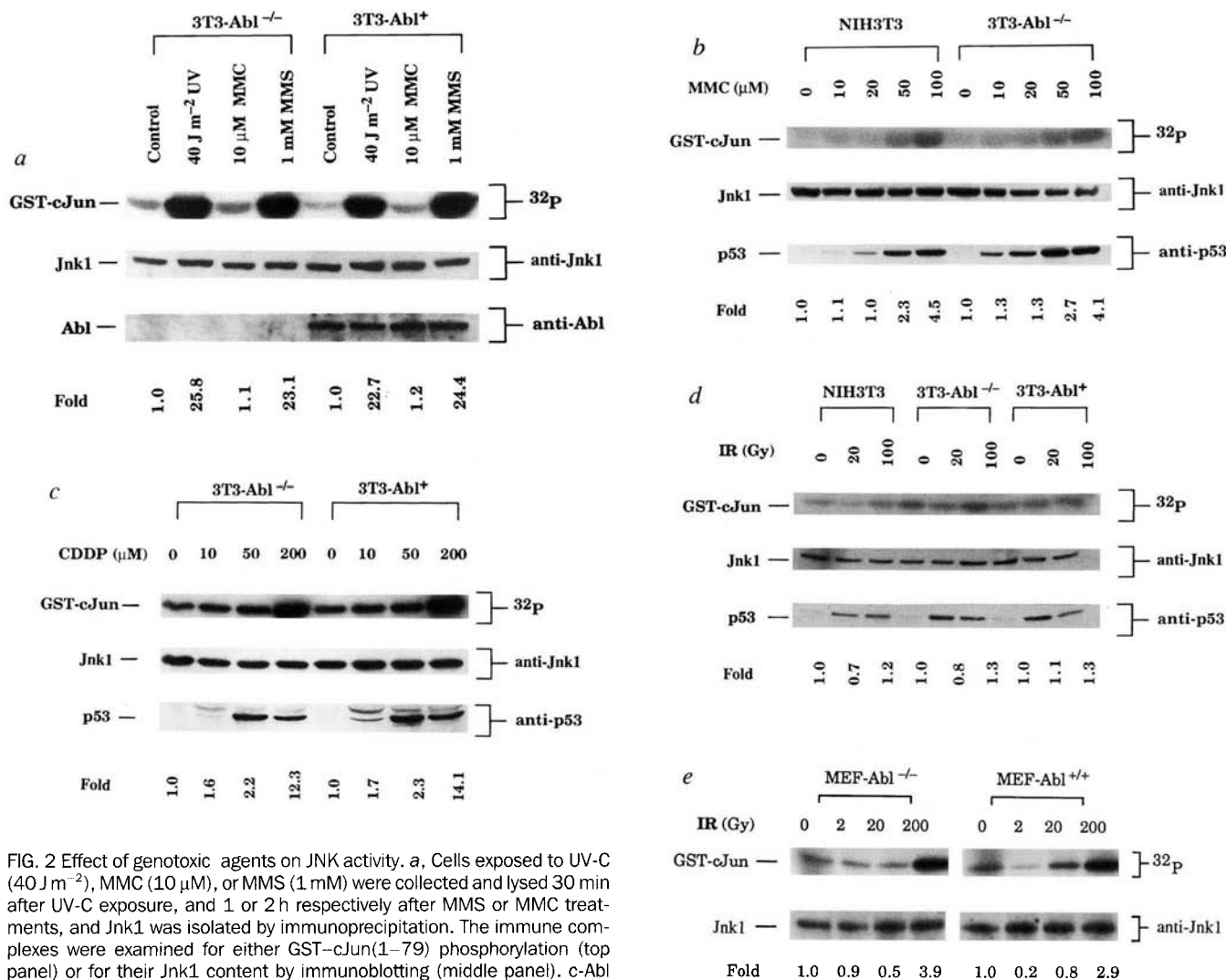
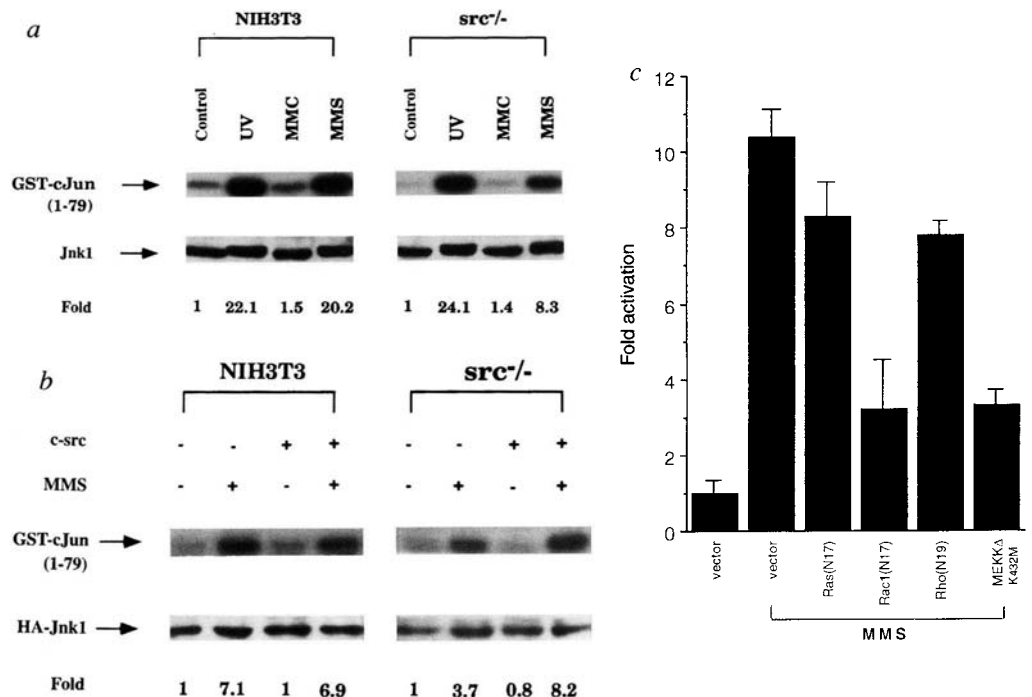


FIG. 2 Effect of genotoxic agents on JNK activity. **a**, Cells exposed to UV-C (40 J m⁻²), MMC (10 μM), or MMS (1 mM) were collected and lysed 30 min after UV-C exposure, and 1 or 2 h respectively after MMS or MMC treatments, and Jnk1 was isolated by immunoprecipitation. The immune complexes were examined for either GST-cJun(1–79) phosphorylation (top panel) or for their Jnk1 content by immunoblotting (middle panel). c-Abl expression was examined by immunoblotting (bottom panel). **b**, **c**, Cells treated with MMC and CDDP were collected 2 h later and the JNK activity and content determined. p53 induction was examined by immunoblotting of cell lysates (collected at 8 h after treatments) with anti-p53 antibody (bottom panel). JNK activity and p53 content were measured at the peak times for each response, which were determined by time course analysis. **d**,

Cells exposed to γ -irradiation were collected 1 h later and the JNK activity and content determined. p53 induction was examined in cells treated in parallel but collected at 2 h after exposing to γ -irradiation. **e**, Mouse embryo fibroblasts (*c-abl*^{-/-} or *c-abl*^{+/+}) were γ -irradiated and collected 1 h later for Jnk1 kinase assays.

FIG. 3 JNK activation by MMS requires c-Src and Rac activities. **a**, Either *c-src*^{-/-} or *c-src*^{+/+} (NIH3T3) immortalized mouse fibroblasts were treated with UV-C or MMS and JNK activity and expression determined. **b**, *c-src*^{-/-} or *c-src*^{+/+} cells were transfected with HA-Jnk1 and c-Src expression vectors and then stimulated with MMS for 1 h. Transfected cells were collected, lysed and HA-Jnk1 isolated by immunoprecipitation and its activity determined by immune-complex kinase assay. HA-Jnk1 expression was determined by immunoblotting. **c**, NIH3T3 cells were cotransfected with HA-Jnk1 expression vector together with Ras(N17), Rac(N17), Rho(N19) or MEKKA (K432M) expression vectors and stimulated with MMS. HA-Jnk1 activity was determined by immune-complex kinase assay and normalized to its expression level, determined by immunoblotting. Results are the average of three separate experiments.



v-Abl contains viral Gag sequences and differs substantially in its subcellular distribution and function from c-Abl²³. Additionally, we found that cotransfection of *c-abl* had no effect on HA-Jnk1 activity (data not shown). Collectively, these results indicate that activation of JNK, c-Abl and p53 represent distinct signalling responses to genotoxic stress.

The activation of JNK by MMS, like the response to ultraviolet light, may not be mediated by DNA damage *per se*. Previous studies suggest that stimulation of AP-1 activity by ultraviolet radiation depends on tyrosine kinases²⁻⁶. Because the response of JNK to either ultraviolet light or MMS is intact in *c-abl*^{-/-} cells, we tested the involvement of other tyrosine kinases. We previously found that ultraviolet irradiation stimulates c-Src kinase, so we tested the response of fibroblasts derived from *c-src*-null mice²⁴. JNK activation by ultraviolet light was normal in *c-src*^{-/-} fibroblasts, but the response to MMS was decreased to about one third of the normal (Fig. 3a). Cotransfection of an HA-Jnk1 vector with a c-Src expression vector did not potentiate the response to MMS in *c-src*^{+/+} cells (NIH3T3), but it restored HA-Jnk1 activation by MMS in *c-src*^{-/-} cells (Fig. 3b). Although previously we found that expression of catalytically inactive v-Src blocks AP-1 induction by ultraviolet radiation, those experiments did not directly examine the involvement of c-Src in JNK activation. In addition, overexpression of catalytically inactive v-Src may affect other cellular targets (for example, other tyrosine kinases) whose activity remains normal in c-Src-deficient cells. The ability of Src to mediate activation of downstream mitogen-activated protein kinases (MAPKs) is dependent on the small GTP-binding proteins Ras and Rac²⁵. HA-Jnk1 activation by MMS was inhibited (by ~70%; Fig. 3c) by expression of the dominant-negative Rac(N17) protein but was not affected by expression of either dominant-negative Ras(N17) or Rho(N19) proteins. Rho is a member of the same group of small GTP-binding proteins as Rac but unlike Rac is not involved in JNK activation²⁵. Rac(N17) had no effect on the activation of c-Abl by MMS (data not shown). These results indicate that the principal pathway by which MMS leads to JNK activation probably requires Src and Rac. A second pathway not involving c-Src or Rac, however, makes an additional contribution to the response to MMS. As MMS is a potent alkylating agent, it may trigger both pathways via the alkylation and inactivation of a negative-regulatory protein.

p53 is an important mediator of cell-cycle arrest and killing in

response to DNA damage¹. c-Abl may also be involved in DNA-damage-induced growth arrest²⁶. Our finding that c-Abl is only activated in S-phase cells suggested that it is unlikely to contribute to a DNA-damage-induced G1 response. We therefore compared the proliferation of *abl*^{-/-} and *abl*⁺ 3T3 cells in the presence of varying MMS concentrations. At 50 μ M MMS, both cell types proliferated as untreated controls (Fig. 4). Cell-cycle analysis showed that 50 μ M MMS induced a transient delay of S-phase entry in both *abl*^{-/-} and *abl*⁺ 3T3 cells (data not shown). At higher concentrations, both cell types were either arrested (as determined by FACS analysis) or dead (based on trypan-blue exclusion). Results were similar with cisplatin or mitomycin C treatments. We also examined the effect of these agents on the proliferation of *c-src*^{-/-} cells and found that whereas the *c-src*^{-/-} cells were more sensitive than the other cell lines to MMS (50 μ M), their sensitivity to mitomycin C and CDDP was not

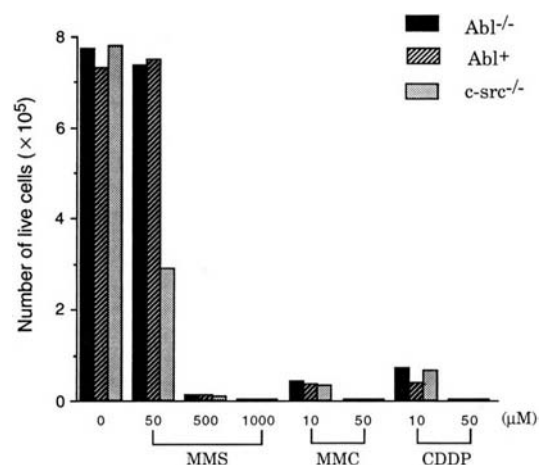


FIG. 4 Growth inhibition and cell killing are not affected by the *c-abl* genotype. 3T3-*Abl*^{-/-} (solid bars), 3T3-*Abl*⁺ cells (hatched bars) and *c-src*^{-/-} cells (stippled bars) were treated with the indicated concentrations of genotoxic agents 24 h after seeding. The initial number of cells seeded was 2×10^4 . After 48 h of treatment, the numbers of live cells were determined by trypan-blue exclusion. Data are representative of two separate experiments.

altered. At concentrations that cause comparable cytotoxic effects, MMS, mitomycin and CDDP vary greatly in their ability to activate JNK (Fig. 2), so it is unlikely that JNK activation is a major determinant of cell killing. On the contrary, the increased sensitivity of *c-src*^{-/-} cells to MMS suggests that JNK activation may give some protection against alkylating agents.

Unlike p53 induction, c-Abl or JNK activation is not a universal response to DNA damage. Different genotoxic agents vary greatly in their ability to activate c-Abl and JNK, yet are effective in inducing p53 accumulation, growth arrest and eventually cell death. Although c-Abl has been implicated in p53-dependent growth arrest in transient assays²⁶, we find that Abl^{-/-} and Abl⁺ fibroblasts showed a similar G1/S delay in response to MMS and ionizing radiation. Furthermore, long-term-survival assays indicate that loss of c-Abl does not contribute to an increased radiosensitivity. Therefore, JNK or c-Abl are unlikely to be general mediators of cell killing or growth arrest by genotoxic agents, at least not in mouse fibroblasts. However, mouse fibroblasts are relatively resistant to the genotoxic effect of the agents tested here²⁷. Determination of the exact role of JNK activation in the cytostatic and apoptotic responses awaits the creation of JNK-null mice and cell lines. □

Methods

Cell culture, treatments and transfections. Polyclonal populations of reconstituted 3T3-Abl⁺ cells and 3T3 Abl-KD (KD, kinase defective) cells were obtained by infection and selection of 3T3-Abl^{-/-} cells with c-abl or kinase-defective c-abl recombinant retroviruses containing the hygromycin-resistance gene. γ -Irradiation was with a ¹³⁷Cs-source emitting a dose of 0.7–7.0 Gy min⁻¹, as calibrated by Frecke dosimetry. Cells were irradiated with UV-C at 40 J m⁻² as described². Mouse embryonic fibroblasts (MEFs) were derived from wild-type and c-abl^{-/-} day-14 embryos and experiments were done on third-passage MEFs. Transient transfections were carried out with lipofectamine (GIBCO BRL) as described²⁸; 0.5 μ g pSR α -HA-Jnk1 and 1 μ g pSR α , pSR α -c-Src, pSR α -Ras(N17), pSR α -Rac(N17), pSR α -Rho(N19) or pSR α -MEKK4(K432M) were used (all plasmids have been described²⁹).

Kinase assay. Abl immunoprecipitation and kinase reactions have been described^{17,28}. KOH-resistant ³²P incorporation into GST-CTD (GST, glutathione-S-transferase) by c-Abl immunocomplexes was determined by phosphorimager and normalized to their c-Abl content. The kinase activity of c-Abl in each sample of untreated cells was given an arbitrary value of 1.0. Note that basal activity levels differed between untreated samples collected at different cell-cycle time points. JNK immunoprecipitations and *in vitro* kinase reactions using GST-tagged amino-terminal c-Jun fragment (1–79) as a substrate were done as described²⁹. GST-cJun phosphorylation was quantified by phosphorimager and normalized to the Jnk1 or HA-Jnk1 content of the immunocomplexes, determined by immunoblotting and chemiluminescence. The data are representative of at least three independent experiments.

Immunoblotting. c-Abl levels were determined by probing with monoclonal anti-Abl antibody (8E9) as described¹⁸. RNAP II immunoprecipitation and P-Tyr blotting were done as described¹⁸. Immunoblotting for Jnk1 used anti-Jnk1 antibody (G151-333.8; Pharmingen) and anti-HA antibody for HA-Jnk1 (ref. 29). Immunoblotting with anti-p53 antibody (Pab240; Pharmingen) was done as described³⁰.

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- Cox, L. & Lane, D. *Bioassays* **17**, 501–508 (1995).
- Devary, Y., Gottlieb, R. A., Smeal, T. & Karin, M. *Cell* **71**, 1081–1091 (1992).
- Devary, Y., Rosette, C., DiDonato, J. A. & Karin, M. *Science* **261**, 1442–1445 (1993).
- Radler-Pohl, A. et al. *EMBO J.* **12**, 1005–1012 (1993).
- Sachsenmaier, C. et al. *Cell* **78**, 963–972 (1994).
- Rosette, C. L. & Karin, M. *Science* (in the press).
- Hibi, M., Lin, A., Smeal, T., Minden, A. & Karin, M. *Genes Dev.* **7**, 2135–2148 (1993).
- Dérjard, B. et al. *Cell* **76**, 1025–1037 (1994).
- Gupta, S., Campbell, D., Dérjard, B. & Davis, R. J. *Science* **267**, 389–393 (1995).
- van Dam, H. et al. *EMBO J.* **14**, 1798–1811 (1995).
- Livingstone, C., Patel, G. & Jones, N. *EMBO J.* **14**, 1785–1797 (1995).
- Whitmarsh, A. J., Shore, P., Sharrocks, A. D. & Davis, R. J. *Science* **269**, 403–407 (1995).
- Cavigelli, M., Dolfi, F., Claret, F. & Karin, M. *EMBO J.* **14**, 5957–5964 (1995).
- Verheij, M. et al. *Nature* **380**, 75–79 (1996).
- Kharbanda, S. et al. *Nature* **376**, 785–788 (1995).
- Kharbanda, S. et al. *J. Biol. Chem.* **270**, 30178–30281 (1995).
- Baskaran, R., Dahmus, M. & Wang, J. Y. J. *Proc. Natl Acad. Sci. USA* **90**, 11167–11171 (1993).
- Baskaran, R., Chiang, G. & Wang, J. Y. J. *Mol. Cell. Biol.* **16**, 3361–3369 (1996).
- Welch, P. J. & Wang, J. Y. J. *Mol. Cell. Biol.* **15**, 5542–5551 (1995).
- Welch, P. J. & Wang, J. Y. J. *Cell* **75**, 779–790 (1993).
- Kallunki, T. et al. *Genes Dev.* **8**, 2996–3007 (1994).
- Renshaw, M. W., Lea-Chou, E. & Wang, J. Y. J. *Curr. Biol.* **6**, 76–83 (1996).

- Wang, J. Y. J. *Curr. Opin. Genet. Dev.* **3**, 35–43 (1993).
- Thomas, J., Soriano, P. & Brugge, J. *Science* **254**, 568–571 (1991).
- Minden, A., Lin, A., Claret, F.-X., Abo, A. & Karin, M. *Cell* **81**, 1147–1157 (1995).
- Yuan, Z.-M. et al. *Nature* **382**, 272–274 (1996).
- Lowe, S. W., Ruley, H. E., Jacks, T. & Housman, D. E. *Cell* **74**, 957–967 (1993).
- Duyster, J., Baskaran, R. & Wang, J. Y. J. *Proc. Natl Acad. Sci. USA* **92**, 1555–1559 (1995).
- Minden, A. et al. *Science* **266**, 1719–1723 (1994).
- Caelles, C., Helmberg, A. & Karin, M. *Nature* **370**, 220–223 (1994).

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20S cyclosome complex formation and proteolytic activity inhibited by the cAMP/PKA pathway

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THE 20S cyclosome complex (also known as the anaphase-promoting complex) has ubiquitin ligase activity and is required for mitotic cyclin destruction^{1–3} and sister chromatid separation^{4,5}. The formation and activation of the 20S cyclosome complex is regulated by an unknown mechanism. Here we show that Cut4 (ref. 6) is an essential component of the cyclosome in fission yeast. Cut4 shares sequence similarity with BimE, a protein that regulates mitosis in *Aspergillus nidulans*^{7–9}. Mutations in *cut4* result in hypersensitivity to cyclic AMP and to stress-inducing heavy metals, inhibition of the onset of anaphase, disruption of the 20S complex, and inhibition of mitotic cyclin ubiquitination. These phenotypes are fully suppressed by cAMP phosphodiesterase and the protein kinase A (PKA) regulatory subunit and weakly suppressed by Sti1 (an activator of the Hsp70 and Hsp90 chaperones^{10,11}). Suppression correlates with the amount of 20S complex, indicating that cyclosome formation and activation is inhibited by the cAMP/PKA pathway.

We first analysed the growth characteristics of a *cut4-533* fission yeast mutant in two culture media, YPD (Fig. 1a) and EMM2 (Fig. 1d) at either 26 °C or 36 °C. The cells grew in EMM2 at both temperatures, and in YPD only at 26 °C. Growth was inhibited by stress-inducing heavy metals, including CdCl₂ at 1 μ M and 10 μ M (Fig. 1f), and NiSO₄ and [Co(NH₃)₆]Cl₂, each at 1 μ M. These compounds induce the formation of abnormal proteins which are eventually degraded by ubiquitin-dependent proteolysis^{12,13}.

We isolated two multicopy suppressors for *cut4-533:Sti1*⁺ encodes a protein with similarity to Sti1 (which activates Hsp70 and Hsp90 chaperones) and *cgs2⁺/pdel⁺* encodes cAMP phosphodiesterase^{14,15}. *Sti1*⁺ weakly suppresses *cut4-533* through an unknown mechanism. *Cgs2*⁺ is a stronger suppressor of *cut4-533* than *sti1*⁺; mutant *cut4* cells carrying a plasmid containing *cgs2*⁺ (pCgs2) grew normally on YPD (Fig. 1c) at 36 °C, whereas those carrying a plasmid containing *sti1*⁺ (pSti1) grew more slowly (Fig. 1b). *Cgs1*⁺, which encodes the regulatory subunit of PKA¹⁴, was also a strong suppressor of *cut4-533* (Fig. 1c). *cut4-533* mutants did not grow at 36 °C in the presence of 10 mM cAMP

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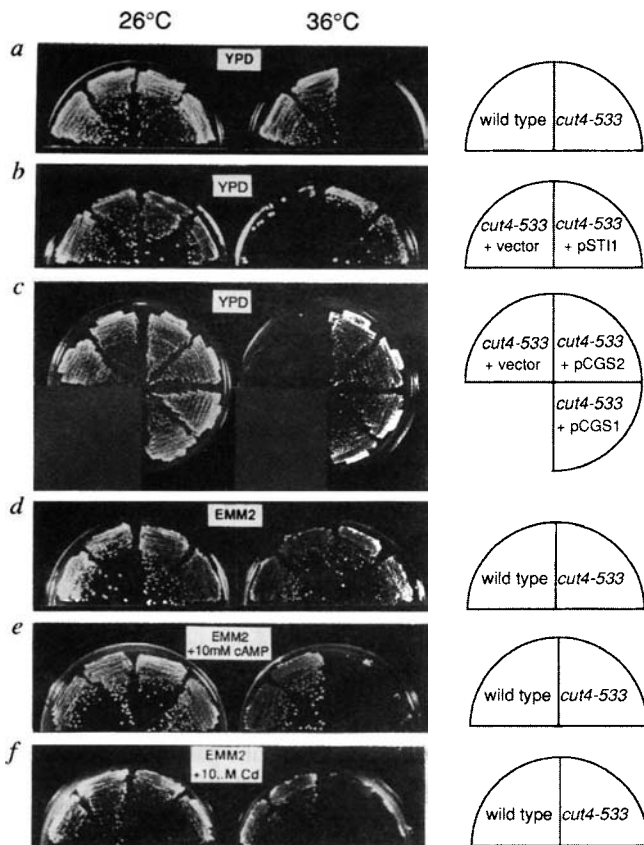


FIG. 1 Growth of wild-type and *cut4-533* mutants. *a*, Wild type and *cut4-533* mutants grown on YPD medium at 26 °C and 36 °C; *b*, *cut4-533* carrying the vector plasmid or pST11 grown on YPD medium at 26 °C and 36 °C; *c*, *cut4-533* carrying the vector plasmids pCGS1 or pCGS2 grown on YPD medium at 26 °C or 36 °C; *d*, wild-type and *cut4-533* mutants grown on EMM2 medium at 26 °C and 36 °C; *e*, wild-type and *cut4-533* mutants grown on EMM2 medium with 10 mM cAMP at 26 and 36 °C; *f*, wild-type and *cut4-533* mutants grown on EMM2 medium with 10 μ M CdCl₂.

(Fig. 1*e*), and mutants carrying a plasmid containing the adenyl cyclase gene or the catalytic subunit of PKA were temperature-sensitive when grown on EMM2 (data not shown). Taken together, these data indicate that inhibition of the cAMP/PKA pathway suppresses the *cut4-533* phenotype.

We cloned the *cut4⁺* gene by chromosome walking (Fig. 2) and found that it encodes a protein of 1,458 amino acids with a predicted relative molecular mass of 166K. It is similar to an open reading frame in budding yeast chromosome XIV (N305, N310), *Aspergillus* BimE (ref. 8), and mouse Tsg24 (ref. 16). Cut4, BimE and Tsg24 are about 40% identical in the central to C-terminal region.

The cellular phenotype of the *cut4-533* mutant in YPD at 36 °C,

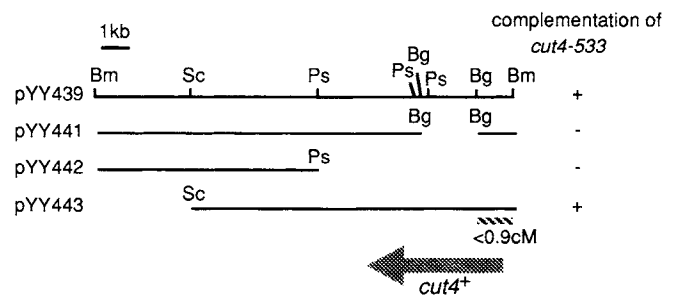


FIG. 2 Cloning of the *cut4⁺* gene. Plasmid pYY439 was obtained from cosmid 813. A subcloned plasmid pYY443 has an open reading frame containing sites essential for complementation.

is similar to that of *cut9* (ref. 17). In *cut4-533* mutants there is a block or delay in the progression from metaphase to anaphase, a high frequency of condensed chromosomes and short spindles (Fig. 3), and an increase of septated cells, many of which exhibit the *cut* (for cell untimely torn) phenotype in the absence of nuclear division. Similar results were obtained when *cut4-533* was cultured in EMM2 containing 10 μ M CdCl₂ or 10 mM cAMP at 36 °C (data not shown). In contrast, the cellular phenotype of *cut4-533* mutants carrying plasmids containing wild-type *cut4⁺* or *cgs2⁺* was identical to that of wild-type cells (data not shown). Cell-cycle analysis indicated that *cut4-533* maintained the checkpoint control when progression through G1 or S phase was inhibited (data not shown).

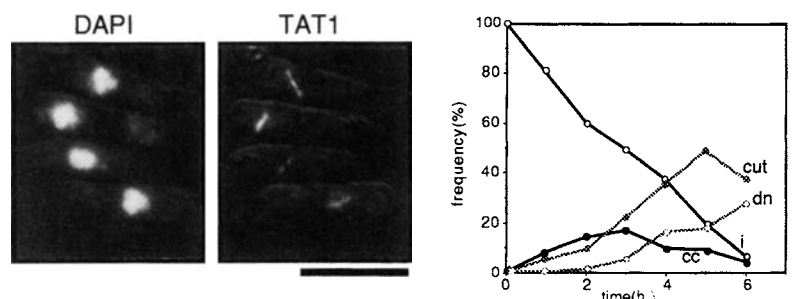
We disrupted the *cut4⁺* gene and performed tetrad dissection of heterozygous diploids. We found that two viable spores in each tetrad were always Ura⁻, indicating that *cut4⁺* is essential for viability. Germinated cells were divided ~3 times and then arrested. Chromosomes in these gene-disrupted cells were condensed with a short spindle (data not shown). 60% of the cells displayed the metaphase phenotype at 30 h after germination. H1 kinase activity was high (Fig. 4), indicating that gene disruption of *cut4⁺* causes a delay or block in exit from mitosis.

We found that Cut4 was a subunit of the 20S cytosome. In sucrose gradients, wild-type Cut4 cosedimented with Cut9 and Nuc2, putative components of the 20S complex^{2,17,18} (Fig. 5*a*). A higher-molecular mass smear is present at the bottom of the gradients (>40S), the origin of which is unknown. Haemagglutinin-tagged Cut4 (HA-Cut4) also sedimented at 20S (Fig. 5*c*).

We then examined whether Cut4 immunoprecipitates with Nuc2 or Cut9. Extracts were prepared from *cut4* null cells carrying a plasmid containing HA-Cut4 next to an inducible promoter¹⁹. The cells were grown in the presence of thiamine and although the promoter was off, a small amount of protein synthesis occurred. HA-Cut4 co-immunoprecipitated with Nuc2 and Cut9 (Fig. 5*d*), indicating that under these conditions, Cut4 interacts with Nuc2 and Cut9.

We next studied formation of the 20S complex in normal *cut4-533* mutants and mutants that were either weakly or strongly

FIG. 3 Cellular phenotype of *cut4-533* cells. *a*, Frequencies (%) of different cell types in YPD medium at 36 °C: (◆) *cut*, cells with the *cut* (cell untimely torn) phenotype; (◇) *dn*, septated cells with a single displaced nucleus; (○) *i*, cells containing an interphase nucleus; (●) *cc*, cells containing condensed chromosomes. *b*, Anti-DNA staining with DAPI and anti-tubulin staining of *cut4-533* cells.



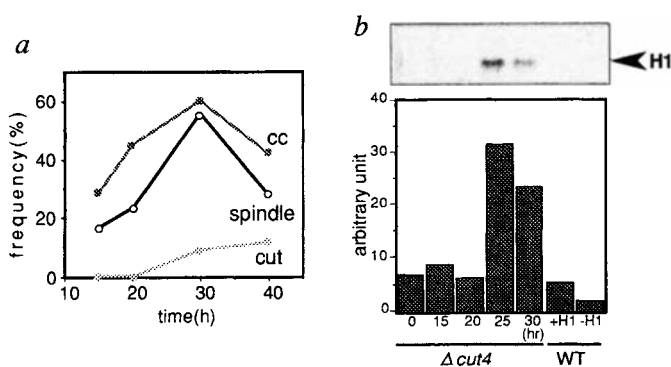


FIG. 4 Frequencies of different cell types and H1 kinase activity in cells with a disrupted *cut4*⁺ gene. **a**, Frequencies (%) of different cell types in YPD medium at 36 °C. (●) cc, cut (abbreviations as for Fig. 3), and (○) spindle, cells containing short spindles. **b**, H1 kinase activity indicated by histogram and autoradiography. In wild-type cells (WT), kinase activity in the presence (+H1) or absence (-H1) of substrate is shown.

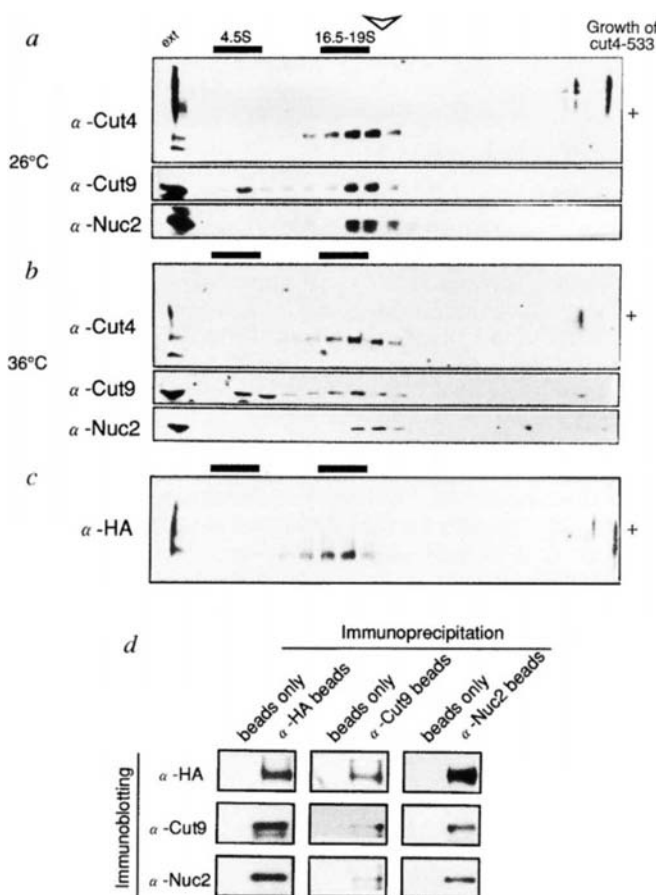


FIG. 5 Cut4 is a component of the 20S cyclosome complex. The 20S complex is indicated by an open arrow. Sucrose gradient centrifugation of extracts from **a**, wild-type cells grown at 26 °C; **b**, wild-type cells grown at 36 °C; and **c**, cells that have a disrupted *cut4*⁺ gene and grown carrying a plasmid containing HA-Cut4 (pHA-CUT4) grown at 33 °C. **d**, Extracts of cells with a disrupted *cut4*⁺ gene carrying pHA-CUT4 were immunoprecipitated by antibodies against HA, Cut9 or Nuc2 Sepharose beads. Immunoprecipitates were western-blotted with antibodies against HA, Cut9 or Nuc2.

suppressed by *still1*⁺ or *cgs2*⁺, respectively. We found that the complex only formed when the mutant was cultured under conditions that favoured growth (compare Fig. 6a and b). Formation of the complex seems to be inversely correlated with the level of suppression: the amount of complex was greater when the mutant carried pCGS2 compared with pSTI1 (compare Fig. 6c and d).

We next analysed whether ubiquitination of mitotic cyclin requires functional Cut4 (Fig. 7). We used the *mts2* mutant, which has ubiquitinated proteins with extended half-lives owing to the presence of a defective 26S proteasome subunit. HA-tagged Cdc13 was immunoprecipitated using an anti-HA antibody. Both ubiquitinated (upper bands) and non-ubiquitinated Cdc13 were detected by western blotting using anti-HA antibody; only ubiquitinated Cdc13 was detected using anti-ubiquitin antibody. *mts2-cut4* double mutants contained less ubiquitinated Cdc13 than *cut4-533* mutants, indicating that ubiquitination of Cdc13 requires Cut4.

We have shown that cAMP and heavy metals inhibit the onset of anaphase in *cut4-533* mutants. *nuc2* and *cut9* mutants do not share this phenotype, indicating that Cut4 plays a distinct role in the regulation of 20S complex formation. The mechanism by which cAMP phosphodiesterase and the regulatory subunit of PKA suppress the *cut4-533* phenotype is unclear. They might stabilize the 20S complex, which could increase ubiquitin-mediated proteolysis of the targets of the 20S complex, such as mitotic cyclins or Cut2 (ref. 5). They may help to eliminate stress-induced abnormal proteins either by activating renaturation or ubiquitin-dependent proteolysis. We found that inhibition of the cAMP/PKA pathway correlates with the presence of the 20S complex. This indicates that PKA directly or indirectly acts on 20S complex formation, probably through Cut4. PKA may negatively regulate type-1 protein phosphatase or Sds23, both of which

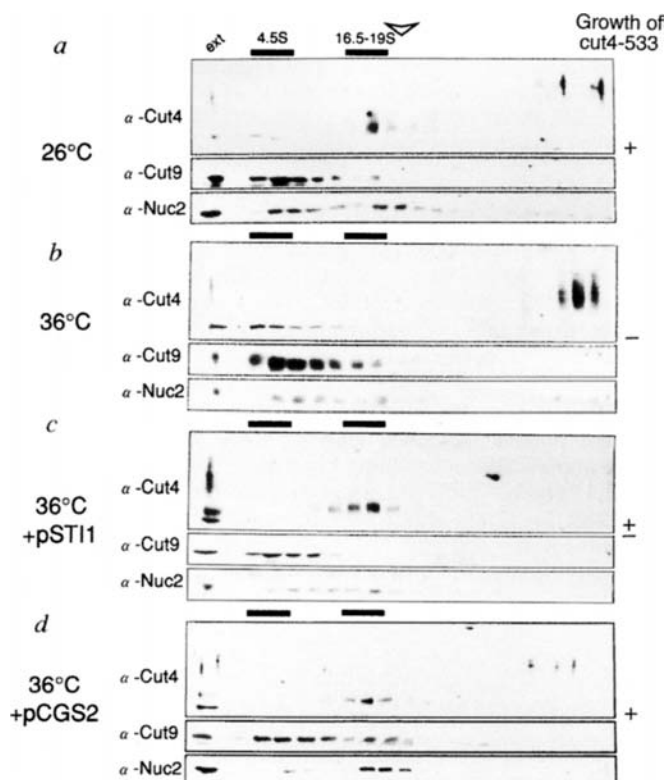


FIG. 6 Formation of the 20S complex in *cut4-533* mutants is inversely correlated with the level of suppression. Sucrose gradient centrifugation of *cut4-533* mutants grown in EMM2 medium containing 10 μM CdCl₂ at **a**, 26 °C, or **b**, 36 °C. Sucrose gradient centrifugation of *cut4-533* mutants (grown in EMM2 medium containing 10 μM CdCl₂) carrying **c**, pSTI1, or **d**, pCGS2. The 20S complex is indicated by an open arrow.

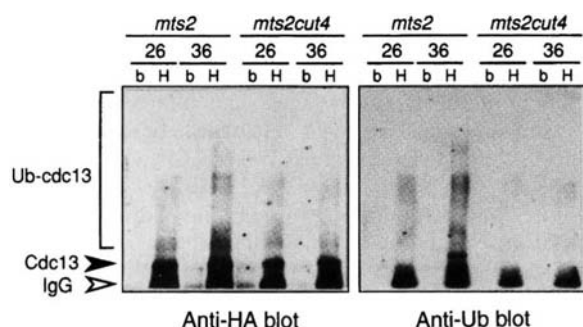


FIG. 7 Ubiquitination of Cdc13 is decreased in the absence of Cut4. *mts2* mutants and *mts2-cut4* double mutants carrying pHA-Cdc13 were grown in YPD at 26 °C or 36 °C. Cdc13 was immunoprecipitated with anti-HA antibodies. Immunoprecipitates were immunoblotted with anti-HA and anti-ubiquitin antibodies.

are important for exit from mitosis and for normal functioning of the 20S complex²⁰. We found that mutations in Cut1 and Cut2 (ref. 5) are also suppressed by pCGS1 and pCGS2 (H. Funabiki, Y.M.Y., D.M. and M.Y., unpublished observation), indicating that not only anaphase proteolysis but also sister chromatid separation may be under the control of the cAMP/PKA pathway. In *Xenopus* eggs, PKA activation is required for the transition from mitosis to interphase²¹. Taken together, our results indicate that the inactivation of PKA, which occurs during prometaphase and metaphase in *Xenopus*, promotes the formation and function of the 20S complex in fission yeast. □

Methods

Cloning of the *sti1*⁺ and *cgs2*⁺/*pde1*⁺ genes. Two classes of plasmids containing *sti1*⁺ and *cgs2*⁺/*pde1*⁺, respectively, were obtained by screening Ts^r transformants of *cut4-533* using an *S. pombe* genomic DNA library⁶.

Cloning of the *cut4*⁺ gene. Genetic analysis showed that *cut4-533* was linked to *cdc13* (~3 cM) on chromosome II. Cosmid clones near *cdc13*⁺ (ref. 22), which complemented *cut4-533*, was subcloned and a minimal functional clone pYY443 was obtained. A *Bgl*III–*Bam*H1 fragment within the insert was integrated into the chromosomes by homologous recombination with the *S. cerevisiae* gene *LEU2*, and was found to be tightly linked to the *cut4-533* locus (less than 0.9 cM).

Disruption of the *cut4*⁺ gene. Gene disruption of *cut4*⁺ was performed by one-step gene replacement²³. A 2.2-kb *Bgl*III fragment in the insert of pYY443 was replaced with the *S. pombe ura4*⁺ (1.8 kb). The resulting plasmid containing the disrupted *cut4* gene was linearized and used for transformation of a diploid 5A1D (*h*⁺/*h*[−] *leu1*/*leu1* *ura4*/*ura4* *ade6-210*/*ade6-216* *his2*⁺). Tetrad analysis of spores generated from heterozygous diploids showed that only two spores were viable and that the viable spores were all Ura[−], indicating that *cut4*⁺ was essential for viability. H1 kinase activity of the germinating cells was measured as described²⁴. Anti-tubulin antibody TAT1 was used for staining microtubules as described²⁵.

Analysis of the epitope-tagged Cut4 protein. The *cut4*-deletion-carrying plasmid pREP41CUT4-HA was constructed as follows: heterozygous Leu[−] diploid 5A1DΔCUT4, with gene disruption in one of the two native *cut4*⁺ genes, was transformed with pREP41CUT4-HA, a plasmid containing the *cut4*⁺ gene tagged with the HA antigen placed under the control of the *nmt* promoter (pREP41). Transformants were sporulated and viable Ura⁺ Leu[−] haploid segregants were selected. *cut4* deletion mutants carrying pREP41-CUT4-HA grew normally even in the presence of thiamine (promoter-repressed state); extracts for immunoprecipitation were prepared from transformants grown in the presence or absence of thiamine. Sucrose gradient centrifugation was performed with modifications^{1,2}. Immunoprecipitation was done as described²⁶ using anti-HA, anti-Nuc2 and anti-Cut9 antibodies.

7. Osmani, S. A., Engel, D. B., Doonan, J. H. & Morris, N. R. *Cell* **52**, 241–251 (1988).
8. Engle, D. B. et al. *J. Biol. Chem.* **265**, 16132–16137 (1990).
9. James, S. W., Mirabito, P. M., Scacheri, P. C. & Morris, N. R. *J. Cell. Sci.* **108**, 3495–3499 (1995).
10. Chang, H.-C. J. & Lindquist, S. *J. Biol. Chem.* **269**, 24983–24988 (1994).
11. Schumacher, R. J. et al. *J. Biol. Chem.* **269**, 9493–9499 (1994).
12. Jungmann, J., Reins, H.-A., Schobert, C. & Jentsch, S. *Nature* **361**, 369–371 (1993).
13. Seufert, W. & Jentsch, S. *EMBO J.* **9**, 543–550 (1990).
14. Devoti, J., Seydoux, G., Beach, D. & McLeod, M. *EMBO J.* **10**, 3759–3786 (1991).
15. Mochizuki, N. & Yamamoto, M. *Mol. Gen. Genet.* **233**, 17–24 (1992).
16. Starborg, M., Brundell, E., Gell, K. & Höög, C. *J. Biol. Chem.* **269**, 24133–24137 (1994).
17. Hirano, T., Hiraoka, Y. & Yanagida, M. *J. Cell Biol.* **106**, 1171–1183 (1988).
18. Samejima, I. & Yanagida, M. *J. Cell Biol.* **127**, 1665–1670 (1994).
19. Maundrell, K. *J. Biol. Chem.* **265**, 10857–10864 (1990).
20. Ishii, T., Kumada, K., Toda, T. & Yanagida, M. *EMBO J.* (in the press).
21. Grieco, D., Porcellini, A., Avedimento, E. V. & Gottesman, M. *Science* **271**, 1718–1722 (1996).
22. Mizukami, T. et al. *Cell* **73**, 121–132 (1993).
23. Rothstein, R. *J. Meth. Enzymol.* **101**, 202–211 (1983).
24. MacNeil, S. A. & Fantes, P. in *The Cell Cycle: A Practical Approach* (eds Fantes, P. & Brooks, R.) 93–125 (Oxford Univ. Press, New York, 1993).
25. Funabiki, H., Hagan, I., Uzawa, S. & Yanagida, M. *J. Cell Biol.* **121**, 961–976 (1993).
26. Harlow, E. & Lane, D. *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1988).

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G2 cyclins are required for the degradation of G1 cyclins in yeast

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PROGRESSION of the eukaryotic cell cycle is controlled by cyclin-dependent kinases (CDKs)¹. Cdc28, the budding yeast homologue of Cdc2 (Cdk1), is required for both the G1/S and G2/M transitions of the cell cycle². The functional specificity of the Cdc28 kinase is determined by its association with G1 or G2 cyclins. Alternation of cell cycle phases is thus mainly due to mechanisms that ensure that one cyclin family succeeds another. Here we show that the G2 cyclins Clb1, Clb2, Clb3 and Clb4 are required for the proteolysis of the G1 cyclins Cln1 and Cln2, providing a mechanism for coupling synthesis of G2 cyclins with the disappearance of G1 cyclins. Our data indicate that this pathway involves the Ubc9 ubiquitin-conjugating enzyme³. The Cdc34 ubiquitin-conjugating activity⁴ may function redundantly with Ubc9, or it may only be involved in Cln1,2 turnover through its role in promoting the degradation of Sic1, a specific inhibitor of Cdc28–Clb complexes⁵.

It has been shown that G1 cyclins inhibit the proteolysis of some G2 cyclins, which enables G1 cyclins to promote accumulation of the G2 cyclins⁶. The G2 cyclins Clb1–Clb4 inhibit the transcription of the *CLN1* and *CLN2* genes^{7,8}, which ensures the rapid loss of the Cln1,2 proteins after the appearance of the CLB proteins only insofar as the Cln1,2 proteins are highly unstable. We therefore investigated whether Cln1,2 protein instability is dependent on G2 cyclins. There are six *CLB* genes in budding yeast which are expressed in three waves through the cell cycle: *CLB5* and *CLB6* expression is activated in late G1 phase^{9–11}, *CLB3* and *CLB4* expression also begins in late G1 phase, but peaks later in the S phase^{12–14}. Finally, *CLB1* and *CLB2* expression is activated appreciably later in the cell cycle and peaks around the time of mitosis^{12–14}. We used pulse-chase labelling of cells, followed by immunoprecipitation of Cln1 and Cln2, in order to determine the

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1. Sudakin, V. et al. *Mol. Biol. Cell* **6**, 185–198 (1995).
2. King, R. W. et al. *Cell* **81**, 279–288 (1995).
3. Tugendreich, S., Tomkiel, J., Earnshaw, W. & Hieter, P. *Cell* **81**, 261–268 (1995).
4. Irniger, S., Platti, S., Michaelis, C. & Nasmyth, K. *Cell* **81**, 269–277 (1995).
5. Funabiki, H. et al. *Nature* **381**, 438–441 (1996).
6. Hirano, T., Funahashi, S., Uemura, T. & Yanagida, M. *EMBO J.* **5**, 2973–2979 (1986).

half-life of the CLN proteins in the wild-type and different mutant yeast strains. These experiments were done under conditions in which *CLN1* and *CLN2* were expressed in their normal chromosomal context in order to avoid possible artefacts arising from overexpression of their gene products.

The half-life of Cln1 and Cln2 in a *clb5,clb6* double mutant (10 min) was the same as in the wild-type strain (Fig. 1) and there was at best a very slight stabilization of Cln1,2 in a *clb3,clb4* double mutant (Fig. 1). As there is an appreciable functional redundancy among the yeast CLB proteins^{5,12,14}, we examined Cln1,2 stability in strains deleted for multiple *CLB* genes. Cln1,2 were significantly stabilized in a *clb1,clb3,clb4* triple mutant (half-life, 30 min) and highly stabilized (half-life, 60 min) in a *clb1,clb3,clb4,clb2^{ts}* quadruple-mutant strain (Fig. 2). The quadruple-mutant cells are almost exclusively in the G2 phase of the cycle (Fig. 2) because the low CLB activity inhibits mitosis⁸. However, stabilization of Cln1,2 in the quadruple mutant is not due to the accumulation of mutant cells in the G2 phase of the cell cycle. Indeed, the CLN proteins remain highly unstable even when they are constitutively expressed throughout the cell cycle^{15–17}. Furthermore, *clb1,clb2* double-mutant cells, and *cdc16-1* mutant cells¹⁸ at 34 °C, are mostly in the G2 phase, and yet Cln1,2 are not stabilized in these cells. Finally, we obtained the same results when we integrated a construction in which *CLN2* is expressed from the weak, constitutive *S. pombe* alcohol dehydrogenase (ADH) promoter¹⁹ in the *cdc16-1*, *clb1,clb2*, and *clb1,clb3,clb4,clb3^{ts}* strains, thereby uncoupling effects of these mutants on *CLN2* transcription from their effects on Cln2 stability (data not shown). We conclude that Clb1–4 are required in a functionally redundant manner for the degradation of Cln1,2.

It is controversial whether the Cdc34 ubiquitin-conjugating enzyme⁴ is required for the degradation of the CLN proteins^{16,20–23}. We verified that Cln1,2 are clearly stabilized in our *cdc34-2* mutant. Moreover, we show here that they are also stabilized in the *cdc4-1* mutant (Fig. 3). Cdc4 is a protein of unknown function which may act together with Cdc34 (refs 5, 23, 24). The simplest interpretation of these results is that the Cdc34 ubiquitin-conjugating complex transfers ubiquitin to Cln1,2 to target their degradation by the 26S proteasome^{20,22,23}. However, given our observation that Clb1–4 are required for Cln1,2 degradation, a less direct action of Cdc4 and Cdc34 in Cln1,2 turnover is possible. Cdc4 and Cdc34 are also required for the degradation of Sic1, a polypeptide inhibitor of Cdc28–Clb kinase complexes^{5,25–27}. Stabi-

lization of Sic1 in the *cdc4* and *cdc34* mutants specifically inhibits Cdc28–Clb complexes within these cells. This inhibition of Cdc28–Clb activity could have the same effect as the mutational inactivation of the *CLB1–4* genes and indirectly lead to the stabilization of the Cln1,2. To distinguish between these two possible mechanisms of action of Cdc4 and Cdc34 in the degradation of Cln1,2, we determined Cln1,2 half-lives in *cdc4,sic1* and *cdc34,sic1* double mutants. If Cdc4 and Cdc34 are directly required for degradation of Cln1,2, then these G1 cyclins should remain stable in *cdc4,sic1* and *cdc34,sic1* double mutants, but pulse-chase experiments showed the converse was the case. Cln1,2 was destabilized when *SIC1* was deleted from the *cdc4* or *cdc34* mutants (Fig. 3). We conclude that Cdc4 and Cdc34 may be required for Cln1,2 degradation only through their action in promoting Sic1 proteolysis. Alternatively, Cdc4 and Cdc34 may function redundantly with an independent degradation pathway that can be activated by the G2 cyclins to promote Cln1,2 turnover. This pathway may involve the Ubc9 ubiquitin-conjugating enzyme, in view of our observation that Cln1,2 are significantly stabilized in the *ubc9-1* temperature-sensitive mutant at the restrictive temperature (Fig. 4a). Ubc9 has been previously implicated in the proteolysis of Clb2 and Clb5 (ref. 3).

A model summarizing our observations and previous results on

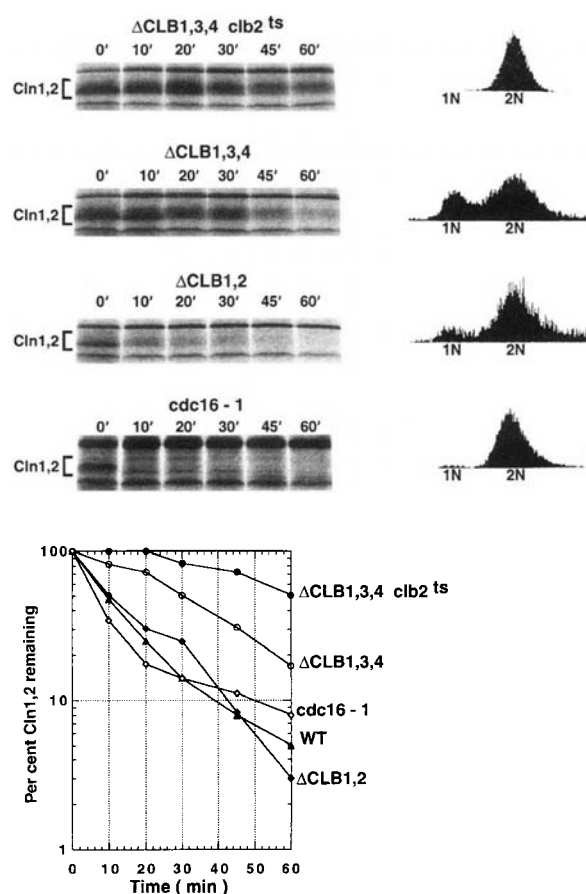


FIG. 2 Cln1,2 are highly stabilized in a *clb1,clb3,clb4,clb2^{ts}* quadruple mutant but only partially stabilized in a *clb1,clb3,clb4* triple-mutant strain; they remain highly unstable in a *clb1,clb2* double-mutant strain and in a *cdc16-1* strain in which cells are also mainly in the G2 phase of the cell cycle. Shown adjacent to the autoradiograms are fluorocytometric analyses¹⁵ of the DNA content in individual cells at the time of pulse labelling. Expression of *CLN1* and *CLN2* is higher in the triple and quadruple *clb* mutant strains because *CLN1* and *CLN2* transcription is de-repressed^{7,8}. This de-repression cannot account for the Cln1,2 stabilization, because such stabilization was not observed even when *CLN1* and *CLN2* were expressed to much higher levels from the *GAL* promoter^{15–17}.

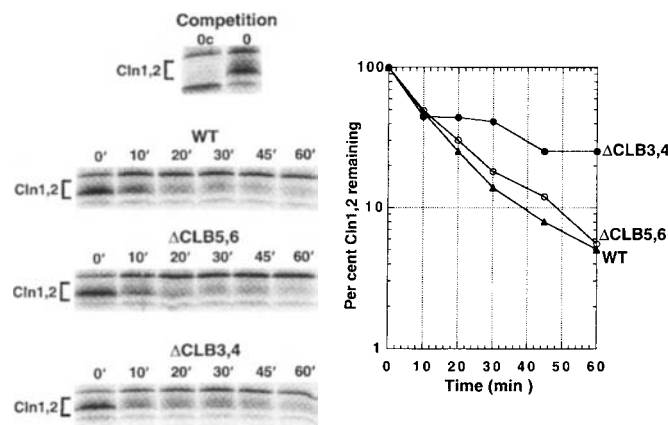


FIG. 1 Cln1,2 remain unstable in *clb5,clb6* and *clb3,clb4* double-mutant yeast strains. Cln1,2 half-lives were determined by pulse-chase analysis as described in Methods. Cln1,2 was identified among the radiolabelled immunoprecipitated proteins using competitive immunoprecipitation of Cln1,2, which resulted from addition to the labelled extracts of 5 μ g unlabelled Cln1 purified from *E. coli* (Competition-0c); these bands were not present when a *cln1,cln2* double-disrupted strain was analysed in the same way (data not shown).

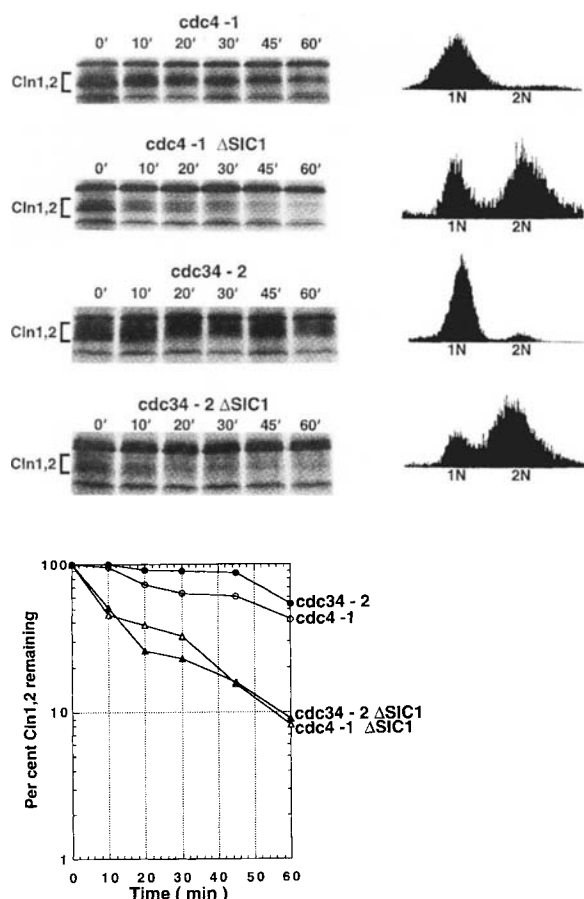


FIG. 3 Cln1,2 are stabilized in *cdc4^{ts}* and *cdc34^{ts}* mutants, but are destabilized in *cdc4^{ts}Δsic1* and *cdc34^{ts}Δsic1* double mutants. The construction and characterization of these mutant strains has been described⁵. Pulse-chase and fluorocytometric analyses were done as before¹⁵, after transferring the temperature-sensitive mutants from 24 to 34 °C (the restrictive temperature) for 4 h.

mechanisms for alternating cell cycle phases is presented in Fig. 4b. Activation of *CLN1* and *CLN2* gene transcription begins at Start in G1 phase. Cdc28–Cln1,2 activity inhibits CLB proteolysis, allowing them to accumulate⁶. Cdc28–Cln activity is also required, together with Cdc4 and Cdc34 for the turnover of Sic1 (refs 5, 27). Degradation of Sic1 relieves the repression of Cdc28–Clb complexes, which can then inhibit the transcription of *CLN1* and *CLN2* (refs 7, 8) and promote Cln1,2 proteolysis through an as-yet undetermined mechanism that may involve Ubc9. This proposed mechanism ensures that the G1 cyclins trigger their own degradation only after they have accumulated sufficiently to perform their physiological functions, notably the induction of Sic1 proteolysis by the Cdc4/Cdc34 pathway^{5,27,28}. Loss of Clb1–4 at mitosis¹³ would allow Cln1,2 to reaccumulate upon transcriptional induction of their genes at Start in the G1 phase of the preceding cell cycle. The model highlights the interplay of proteolytic events that control the timing and alternation of the cell cycle phases. □

Methods

Yeast strain constructions. The *clb5,clb6* strain was made by precisely deleting the *CLB5* and *CLB6* coding sequences from the wild-type yeast strain CMY395 by consecutive transformation with *clb5::HIS3* and *clb6::URA3^K* deletion cassettes synthesized by the polymerase chain reaction (PCR)²⁹. The *clb3,clb4* strain was made from CMY395 by disrupting *CLB3* with a *clb3::TRP1* cassette¹³ and deleting *CLB4* with a *clb4::HIS3* deletion cassette prepared by

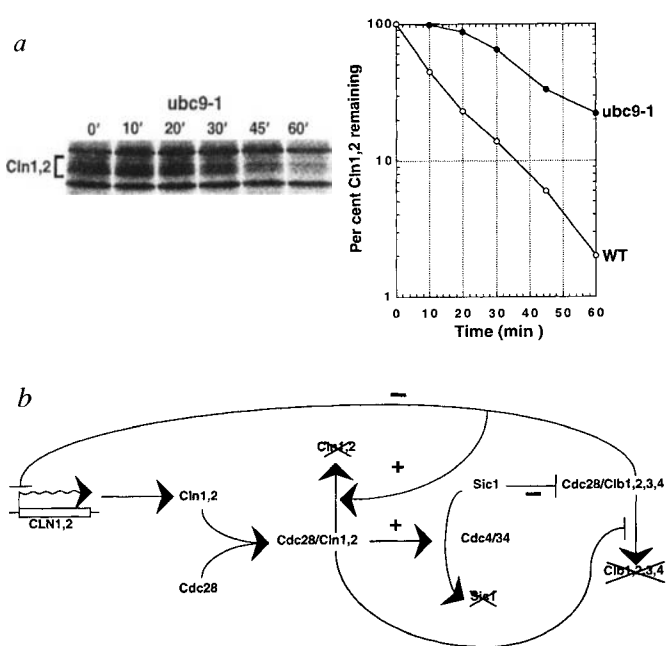


FIG. 4 *a*, Cln1,2 are stabilized in the *ubc9-1^{ts}* mutant. Pulse-chase analysis was carried out as for Fig. 1, except that the *ubc9-1* and congenic wild-type strains were transferred to 35 °C for 4 h before pulse labelling. *b*, Model summarizing the mechanisms known to ensure that cyclin families alternate through the cell division cycle.

PCR²⁹. The *clb1,clb2* double mutant was obtained as a meiotic segregant of a cross between a CMY395 strain deleted for *CLB1* with a *clb1::HIS3* deletion cassette obtained by PCR²⁹ and a congenic YPH499 strain containing a *clb2::LEU2* disruption¹³. The *clb1,clb3,clb4* triple and *clb1,clb3,clb4,clb2^{ts}* quadruple mutants have been described⁸.

Pulse-chase analysis. Cln1,2 half-lives were determined by pulse-chase analysis as described¹⁵. Briefly, yeast cells were pulse-labelled with ³⁵S-methionine/cysteine for 10 min and then incubated for various times with cold methionine/cysteine. Temperature-resistant strains were grown at 30 °C, and temperature-sensitive mutants were transferred from 23 °C to their minimum restrictive temperature (34–35 °C) for 4 h before pulse-chase analyses. Protein extracts were prepared from radiolabelled cells by glass-bead breakage, and Cln1 and Cln2 were immunoprecipitated with a rabbit polyclonal anti-Cln1 antiserum which also reacts with the highly similar Cln2. Immunoprecipitated proteins were electrophoresed in SDS–polyacrylamide gels and the radioactivity in the Cln1,2 bands was quantified by PhosphorImager analysis.

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1. Nigg, E. A. *Bioessays* **17**, 471–480 (1995).
2. Nasmyth, K. *Curr. Opin. Cell Biol.* **5**, 166–179 (1993).
3. Seufert, W., Futcher, B. & Jentsch, S. *Nature* **373**, 78–81 (1995).
4. Goebel, M. G. et al. *Science* **241**, 1331–1335 (1988).
5. Schwob, E., Böhm, T., Mendenhall, M. & Nasmyth, K. *Cell* **79**, 233–244 (1994).
6. Amon, A., Imiger, S. & Nasmyth, K. *Cell* **77**, 1037–1050 (1994).
7. Koch, C., Schleiffer, A., Ammerer, G. & Nasmyth, K. *Genes Dev.* **10**, 129–141 (1996).
8. Amon, A., Tyers, M., Futcher, B. & Nasmyth, K. *Cell* **74**, 993–1007 (1993).
9. Kuhne, C. & Linder, P. *EMBO J.* **12**, 3437–3447 (1993).
10. Schwob, E. & Nasmyth, K. *Genes Dev.* **7**, 1160–1175 (1993).
11. Epstein, C. B. & Cross, F. R. *Genes Dev.* **6**, 1695–1706 (1992).
12. Fitch, I. et al. *Mol. Biol. Cell* **3**, 805–818 (1992).
13. Grandin, N. & Reed, S. I. *Mol. Cell. Biol.* **13**, 2113–2125 (1993).
14. Richardson, H., Lew, D. J., Henze, M., Sugimoto, K. & Reed, S. I. *Genes Dev.* **6**, 2021–2034 (1992).
15. Barral, Y., Jentsch, S. & Mann, C. *Genes Dev.* **9**, 399–409 (1995).
16. Salama, S. R., Hendricks, K. B. & Thorne, J. *Mol. Cell Biol.* **14**, 7953–7966 (1994).
17. Wittenberg, C., Sugimoto, K. & Reed, S. I. *Cell* **62**, 225–237 (1990).
18. Imiger, S., Piatti, S., Michaelis, C. & Nasmyth, K. *Cell* **81**, 269–278 (1995).
19. Nasmyth, K. & Dirick, L. *Cell* **66**, 995–1013 (1991).
20. Yaglom, J. et al. *Mol. Cell. Biol.* **15**, 731–741 (1995).
21. Tyers, M., Tokiwa, G., Nash, R. & Futcher, B. *EMBO J.* **11**, 1773–1784 (1992).
22. Deshaies, R. J., Chau, V. & Kirschner, M. *EMBO J.* **14**, 303–312 (1995).
23. Willems, A. R. et al. *Cell* **86**, 453–463 (1996).
24. Bai, C. et al. *Cell* **86**, 263–274 (1996).
25. Mendenhall, M. D. *Science* **259**, 216–219 (1993).

26. Nugroho, T. T. & Mendenhall, M. D. *Mol. Cell. Biol.* **14**, 3320–3328 (1994).
 27. Schneider, B. L., Yang, Q. H. & Futcher, A. B. *Science* **272**, 560–562 (1996).
 28. Tyers, M. *Proc. Natl Acad. Sci. USA* **93**, 7772–7776 (1996).
 29. Baudin, A., Ozier-Kalogeropoulos, O., Denouel, A., Lacroute, F. & Cullin, C. *Nucleic Acids Res.* **21**, 3329–3330 (1993).

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Cell-cycle related regulation of poly(A) polymerase by phosphorylation

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THE poly(A) tail found on almost all eukaryotic messenger RNAs^{1,2} is important in enhancing translation initiation and determining mRNA stability^{3,4}. Control of poly(A)-tail synthesis thus has the potential to be a key regulatory step in gene expression and is indeed known to be important during early development in many organisms⁵. To study a possible basis for such regulation, we examined phosphorylation of poly(A) polymerase (PAP) by p34^{cdc2}/cyclin B (maturation/mitosis-promoting factor, MPF). We show here that PAP can be phosphorylated *in vivo* and *in vitro* by MPF. Consistent with this, PAP becomes hyperphosphorylated both during meiotic maturation of *Xenopus laevis* oocytes and in HeLa cells arrested at M phase, times in the cell-cycle when MPF is known to be active^{6,7}. We show further that hyperphosphorylation by MPF dramatically reduces the activity of purified PAP, and that PAP isolated from mitotic HeLa cells is similarly inhibited by hyperphosphorylation. This repression probably contributes to the well established reductions in poly(A)⁺ RNA and/or protein synthesis known to occur in M-phase cells^{8–15}.

A recombinant baculovirus expressing bovine PAP I (ref. 16) with an amino-terminal histidine tag (Fig. 1) was constructed and used to infect sf21 cells. PAP I purified from the infected cells was subjected to gel electrophoresis and analysed by silver staining and western blotting, revealing four species with apparent relative molecular masses of about 80K, 85K, 90K and 95K (Fig. 2a, lanes 1 and 3). PAP I produced in bacteria (see below) or translated *in vitro* (not shown) migrated as a single 80K species, suggesting that the multiple forms resulted from post-translational modification. In contrast, when sf21 cells were coinfecting with recombinant baculoviruses expressing PAP I and the two subunits of MPF, p34^{cdc2} and cyclin B, the multiple forms of PAP I were completely converted to a single lower-mobility species (Fig. 2a: compare lanes 1 and 3 with 2 and 4). Confirming that the mobility changes were the result of phosphorylation, phosphatase treatment converted all of the low-mobility species to a single 80K form (Fig. 2b). To prove that the putative hyperphosphorylation was the direct consequence of phosphorylation by MPF, we tested the ability of the purified kinase to phosphorylate purified PAP I *in vitro* (Fig. 2c). MPF again converted the multiple forms of PAP I to the hyperphosphorylated form. CDK-specific inhibitors p21/Waf1 (ref. 17) and olomoucine¹⁸ inhibited the conversion, providing

strong evidence that phosphorylation was due to MPF. We previously described two full-length forms of PAP complementary DNA which arise from alternative splicing^{16,19} (Fig. 1). The second form (PAP II) may in fact be the most abundant, as most cDNAs isolated by others encode PAP II-related isoforms^{20–22}. A baculovirus encoding His-tagged PAP II was constructed and used in experiments similar to those described above. PAP II purified from singly infected cells migrated primarily as two species of about 100K and 105K, but overexpression of MPF again resulted in a single, lower-mobility, hyperphosphorylated species of about 110K (Fig. 2a, lanes 5–8). We conclude that MPF can phosphorylate PAP *in vivo* and *in vitro*.

We next wished to obtain evidence that hyperphosphorylation of PAP occurs naturally *in vivo* at times when MPF is active. Two well studied examples are during oocyte maturation in frogs and mitosis in somatic cells. We first tested whether HeLa cells arrested at M phase exhibit a change in the phosphorylation state of PAP. Western blot analysis was performed with PAP isolated from whole-cell extracts of asynchronous cells and cells arrested at metaphase with nocodazole (Fig. 3a). Multiple forms of PAP were detected in the asynchronous cells (lane 1), and the pattern was similar to that previously reported²¹ and to that observed with PAP II extracted from recombinant baculovirus-infected sf21 cells (Fig. 2a). Faint, higher-mobility species were also detected on longer exposures, which probably correspond to PAP I isoforms (see also Fig. 3a, lanes 3 and 5). These forms were all greatly reduced or absent in the M-phase PAP preparation, and a low-mobility species of about 110K was the major isoform detected (lane 2). Phosphatase treatment of the two PAP preparations eliminated the low-mobility forms and in each case gave rise to higher-mobility species (Fig. 3a, lanes 3–6), confirming that the reduced mobility of the slow forms was due to phosphorylation. These results provide strong evidence that PAP becomes hyperphosphorylated during the M phase of the cell cycle, probably by MPF.

PAP becomes hyperphosphorylated during meiotic maturation of *X. laevis* oocytes, reaching maximal levels in unfertilized eggs (ref. 22, and our unpublished results). To test whether this involves phosphorylation by MPF, a PAP I triple mutant containing serine-to-alanine changes at all three cyclin-dependent kinase consensus sites (cdk⁻ PAP) was constructed. Wild-type and cdk⁻ PAP were first expressed in *Escherichia coli*, and the purified, unmodified PAPs used in *in vitro* kinase assays with MPF and [γ -³²P] ATP. Wild-type, but not cdk⁻ PAP, was phosphorylated (Fig. 3b), confirming that one or more of the cyclin-dependent kinase sites are targets of MPF. Note that phosphorylation of wild-type *E. coli* PAP induced only a small mobility shift, in contrast to that observed with baculovirus-expressed PAP (Fig. 2c). This probably reflects the fact that PAP is phosphorylated at a number of additional sites (see below), and modification of

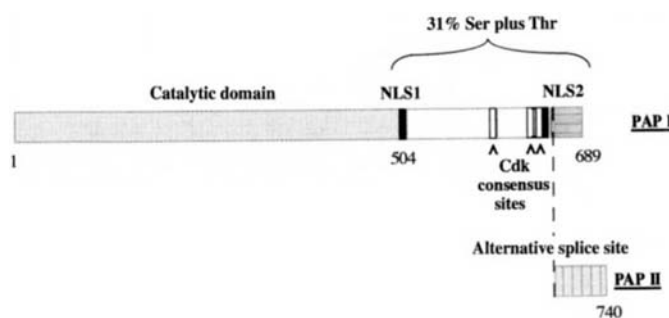


FIG. 1 Structural features of bovine poly(A) polymerase. Schematic diagram of PAP depicting the catalytic region²⁹, the S/T-rich region encompassing the two nuclear localization signals (NLS 1 and 2) and the three cyclin-dependent kinase consensus sites^{16,30}. Also shown is the position of an alternative splice site used to generate PAP II.

some or all of these is necessary for the full mobility shift. Wild-type and cdk^- PAP mRNAs were then synthesized and injected into oocytes. In the absence of progesterone, neither form was detectably modified (Fig. 3c). Following maturation, both became phosphorylated, but phosphorylation of cdk^- PAP was significantly reduced relative to wild-type (Fig. 3c; compare lanes 2 and 4). These results indicate that one or more of the cyclin-dependent kinase consensus sites is indeed phosphorylated during maturation, although a number of additional sites are modified as well.

We next tested whether phosphorylation by MPF affects PAP activity. Underphosphorylated and MPF-hyperphosphorylated PAP I and II purified from recombinant baculovirus-infected sf21 cells were quantified (see Methods) and their activities compared in specific and nonspecific polyadenylation assays¹⁶. Specific assays require the polyadenylation signal sequence AAUAAA on the RNA, as well as cleavage-polyadenylation specificity factor CPSF^{23,24}; nonspecific assays are done in the presence of Mn^{2+} , which relieves the requirements for CPSF and AAUAAA. Strikingly, the activity of the hyperphosphorylated forms of both PAP I (Fig. 4a) and II (results not shown) was

greatly reduced when compared with the underphosphorylated forms. That inhibition was detected in nonspecific assays indicates that repression was due to an intrinsic alteration of PAP. To determine whether the reduced activity of hyperphosphorylated PAP required phosphorylation of one or more of the three consensus cyclin-dependent kinase sites, a recombinant baculovirus expressing cdk^- PAP was constructed. Mutation of the cyclin-dependent kinase sites by itself had no effect on activity, as wild-type and cdk^- PAPs had equal activities (Fig. 4, and data not shown). However, in stark contrast to the repression seen with wild-type PAP purified from triply infected cells, cdk^- PAP I activity was unaffected by coinfection with $p34^{cdc2}$ and cyclin B viruses (Fig. 4b; compare lanes 1–5 with lanes 6–10), confirming that phosphorylation of one or more cyclin-dependent kinase consensus sites is necessary for MPF-induced repression.

If the inhibition of PAP by MPF hyperphosphorylation is physiologically relevant, then hyperphosphorylated PAP isolated from M-phase cells should display significantly reduced activity. To test this, PAP was partially purified from HeLa cells arrested in M phase with nocodazol and from mock-treated cells. The phosphorylation status of the two PAP preparations is shown in Fig. 3a, and Fig. 4c compares their activities in specific polyadenylation assays. Remarkably, the activity of the M-phase hyperphosphorylated PAP was greatly reduced relative to the control PAP (compare lanes 2–4 with 5–7). The inhibition was in fact due to hyperphosphorylation, as phosphatase treatment (Fig. 3a) resulted in very strong activation of the M-phase PAP

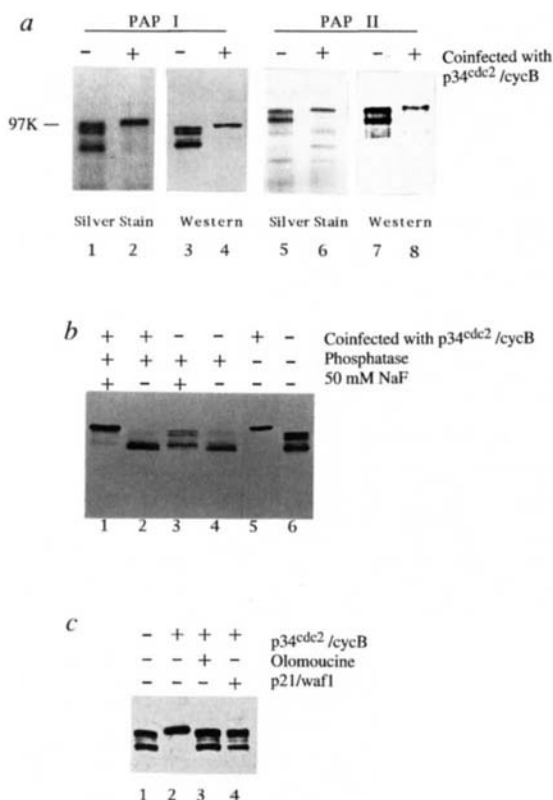


FIG. 2 MPF induces hyperphosphorylation of PAP *in vivo* and *in vitro*. **a**, Recombinant baculovirus PAP is hyperphosphorylated by coinfection with MPF viruses. Recombinant bovine PAP was purified from sf21 cells infected with a PAP I baculovirus (lanes 1 and 3), a PAP II virus (lanes 5 and 7) and PAP I or II virus coinfecting with human $p34^{cdc2}$ and cyclin B viruses (lanes 2 and 4 or 6 and 8, respectively). Proteins were resolved on polyacrylamide SDS-gels and analysed by silver stain (lanes 1, 2, 5 and 6) or western blot (lanes 3, 4, 7 and 8). The position of a 97K size marker is shown. **b**, Underphosphorylated and hyperphosphorylated PAP can be dephosphorylated by potato acid phosphatase. PAP I, underphosphorylated (lanes 3 and 4) or hyperphosphorylated (lanes 1 and 2), was incubated with potato acid phosphatase in the absence (lanes 2 and 4) or presence (lanes 1 and 3) of phosphatase inhibitor. Untreated hyperphosphorylated (lane 5) and underphosphorylated (lane 6) PAP are also shown. Proteins were analysed by western blotting. **c**, PAP can be phosphorylated by MPF *in vitro*. PAP I was incubated with either boiled MPF (lane 1) or native MPF alone (lane 2) or plus the cdk inhibitors olomoucine (lane 3) or p21/Waf1 (lane 4). Proteins were analysed by western blotting.

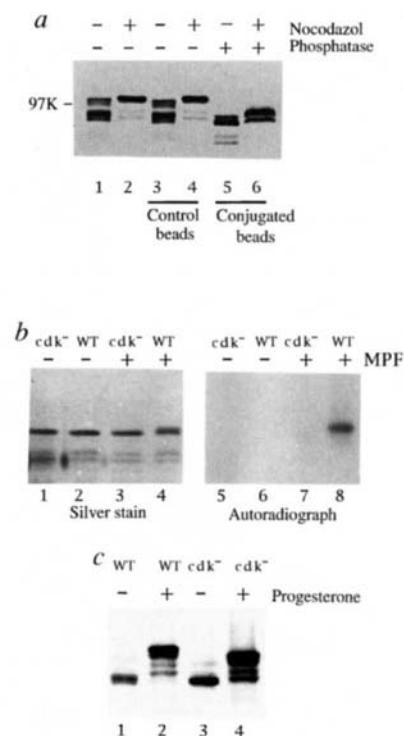


FIG. 3 Poly(A) polymerase is hyperphosphorylated during M phase *in vivo*. **a**, HeLa cells arrested in mitosis accumulate hyperphosphorylated PAP. PAP fractions prepared from asynchronous (lane 1) or M-phase-arrested (lane 2) HeLa cells were incubated with control agarose beads (lane 3 and 4) or alkaline phosphatase-conjugated beads (lanes 5 and 6) and analysed by western blotting. **b**, Wild-type but not cdk^- PAP I is phosphorylated *in vitro* by MPF. Wild-type (lanes 2, 4, 6 and 8) and cdk^- mutant (lanes 1, 3, 5 and 7) PAP I from *E. coli* were incubated with [γ -³²P] ATP in the absence (lanes 1, 2, 5 and 6) or presence (lanes 3, 4, 7 and 8) of MPF. Lanes 5–8 show the autoradiograph of lanes 1–4. **c**, PAP is phosphorylated on CDK consensus sites and other sites during oocyte maturation. Oocytes were injected with wild-type (lanes 1 and 2) or cdk^- mutant (lanes 3 and 4) PAP I mRNA. Oocytes were untreated (lanes 1 and 3) or treated with progesterone (lanes 2 and 4), lysed and PAP immunoprecipitated using anti-PAP antisera and analysed by SDS-PAGE.

(Fig. 4d; compare lanes 2 and 3), but only slightly affected the activity of the control enzyme (Fig. 4d; compare lanes 4 and 5 and 6 and 7). These results strongly suggest that MPF hyperphosphorylation inhibits PAP during M-phase of the cell cycle.

Our data have provided evidence that MPF-induced hyperphosphorylation of PAP occurs in two cellular environments: unfertilized eggs and mitotic somatic cells. In both cases there are considerable data that are consistent with the idea that polyadenylation is inhibited in such cells. In unfertilized eggs of several species, including *X. laevis*, a sharp decline in the accumulation of poly(A) (refs 10, 11) and in poly(A) synthesis^{13–15} has been documented. Our data suggest that this decline results directly from inhibition of PAP by MPF. Intriguingly, this inhibition is reversed following fertilization, when poly(A) synthesis resumes^{10,11,14,15}, PAP becomes partially dephosphorylated²², and MPF levels decline²⁵. The hyperphosphorylation and inhibition of PAP isolated from M-phase HeLa cells indicates that the enzyme is also downregulated during mitosis. It has been well established

that cells entering mitosis show a general repression of RNA and protein synthesis^{8,9,12}, and inhibition of PAP activity could contribute to this in several ways. Messenger RNAs synthesized without a poly(A) tail would probably be rapidly degraded, and those that survive would be poor templates for translation. Following disintegration of the nuclear membrane, inhibition of PAP would prevent possible inappropriate poly(A)-tail lengthening of pre-existing cytoplasmic mRNAs by PAP released from the nucleus. Whatever the precise mechanisms, our data suggest that inhibition of PAP by MPF-catalysed phosphorylation is an important aspect of the silencing of gene expression that occurs in M-phase cells. □

Methods

Preparation of PAPs. PAP was purified from 10^9 Sf21 cells infected with 1 PFU per cell PAP I, II or cdk⁻ PAP recombinant baculoviruses or with 1 PFU per cell of a PAP virus plus 3 PFU per cell of recombinant human p34^{cdc2} and cyclin B viruses. After 40 h, cells were lysed in 10 ml of 50 mM Tris, pH 8.0, 150 mM NaCl, 40 mM imidazole and 0.1% aprotinin, 10 mM benzamide, 30 μ g ml⁻¹ leupeptin, 1 μ g ml⁻¹ bacitracin, 10 μ g ml⁻¹ α -2-macroglobulin and 0.35 mM phenylmethylsulphonyl fluoride (PMSF). Lysates were spun for 15 min at 37,000g, supernatants collected and rocked for 1 h with 0.25 ml Ni²⁺-NTA agarose (Qiagen). Beads were washed 5 times with RIPA buffer (150 mM NaCl, 50 mM Tris, pH 7.2, 1.0% Nonidet P-40, 2% sodium deoxycholate, 0.1% SDS) and eluted with 0.2 ml of 40 mM HEPES, pH 7.5, 20% glycerol, 100 mM imidazole, plus protease inhibitors. To produce PAP and cdk⁻ PAP in *E. coli*, N-terminally His-tagged PAPs were expressed for 3 h in 250 ml LB buffer plus 25 mg ml⁻¹ rifampicin. Cells were pelleted, washed twice in TEK (20 mM Tris-Cl, pH 7.5, 100 mM KCl, 1 mM EDTA), resuspended in 2 ml TEK, 0.5% Nonidet P-40 plus protease inhibitors, sonicated and PAP purified as above. Preparations were equilibrated with buffer D (20 mM HEPES-KOH (pH 7.9), 50 mM (NH₄)₂SO₄, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 10% glycerol) on a Fast Desalting column (Smart System, Pharmacia). For the purification of HeLa PAP, cells in suspension (3 l) were grown to a density of 5×10^5 per ml. For M-phase PAP, nocodazol (Sigma) was added to a final concentration of 40 ng ml⁻¹. After 24 h, 98% of the treated cells were arrested in M phase, as determined by fluorescence-activated cell sorting (FACS) analysis (results not shown), whereas the untreated cells showed a normal cell-cycle distribution. Cells were collected and whole-cell extracts were prepared as described previously²⁶. The (NH₄)₂SO₄ precipitate was suspended in 15 ml of buffer D and fractionated on a Superose 6 column (Pharmacia). PAP concentrations were determined by western and slot blot analysis using affinity-purified PAP antibodies.

Phosphorylation and dephosphorylation of PAP. Baculovirus-produced PAP I was incubated for 30 min at 37 °C with 5 μ l potato acid phosphatase (Boehringer) in 100 mM NaCl, 50 mM PIPES, pH 6.0, and 0.035 mM PMSF, in the presence or absence of 50 mM NaF. PAP preparations (50 μ l) from HeLa cells were incubated in buffer D plus 1 mM MgCl₂ and 1 mM DTT at 37 °C with 20 μ l of agarose beads or alkaline phosphatase-conjugated agarose beads (25 units). Beads were removed by brief centrifugation and 5–15 μ l of the indicated PAP preparation was used in polyadenylation assays. For kinase assays, 250 ng of wild-type or cdk⁻ PAP produced in *E. coli* or baculovirus was incubated in 50 μ l kinase buffer (5 mM MnCl₂, 0.1 mM DTT, 25 mM HEPES, pH 7.5, and 100 μ M ATP) at 30 °C for 1 h, in the presence of 50 ng boiled or native purified MPF²⁷ alone or with 50 ng p21/Waf1 or 10 μ g ml⁻¹ olomoucine.

Oocyte injections. Groups of 10 oocytes cytoplasmically injected with 50 ng bovine PAP I or cdk⁻ PAP mRNA (including a poly(A) tail of 40 residues) synthesized *in vitro* and labelled with ³⁵S methionine were processed as described²⁸. PAP was immunoprecipitated from lysates with anti-PAP polyclonal antisera. Immunoprecipitates were resolved on a 7.5% polyacrylamide gel and exposed to film.

Polyadenylation assays. Polyadenylation assays were performed as described¹⁶. In specific assays, increasing amounts of PAP were added to reaction mixtures containing 2.0 μ l of calf thymus MonoQ-purified CPSF²⁴ and ³²P-labelled SV40 late pre-RNA. In nonspecific assays, reactions contained 0.5 mM Mn²⁺ instead of Mg²⁺ and no CPSF. RNA products were analysed on a 5% polyacrylamide–8M urea gel.

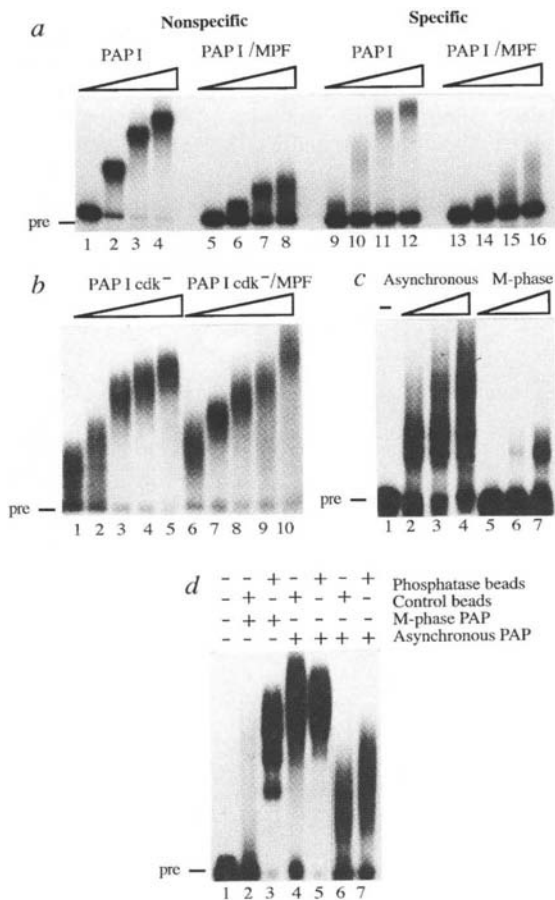


FIG. 4 MPF-induced hyperphosphorylation inhibits PAP. **a**, Coinfection with PAP baculoviruses plus MPF viruses represses PAP. Baculovirus-produced underphosphorylated (3, 12, 48 and 96 ng; lanes 1–4, 9–12) and hyperphosphorylated PAP (lanes 5–8, 13–16) were tested in nonspecific (**a**, lanes 1–8) and specific (lanes 9–16) polyadenylation assays. **b**, cdk⁻ PAP is resistant to MPF-induced repression. cdk⁻ PAP I (10, 20, 30, 40 and 50 ng) was tested in nonspecific polyadenylation assays purified from singly infected (lanes 1–5) and MPF coinfecting (lanes 6–10) sf21 cells. **c**, Repression of M-phase PAP. Partially purified PAPs (5–15 μ l) from asynchronous (lanes 2–4) or M-phase (lanes 5–7) HeLa cells was assayed in a specific polyadenylation assay. Lane 1, precursor RNA with CPSF in the absence of PAP. **d**, Phosphatase treatment specifically activates M-phase PAP. Specific polyadenylation assay with partially purified PAPs from asynchronous (lanes 4–7) or M-phase (lanes 2 and 3) HeLa cells, pretreated with either control agarose beads (lanes 2, 4 and 6) or alkaline phosphatase beads (lanes 3, 5 and 7). Lanes 2–5 contained 15 μ l PAP; lanes 5–7, 5 μ l PAP.

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- Keller, W. *Cell* **81**, 829–832 (1995).
- Manley, J. L. *Curr. Opin. Genet. Dev.* **5**, 222–228 (1995).
- Beelman, C. A. & Parker, R. *Cell* **81**, 179–183 (1995).
- Curtis, D., Lehmann, R. & Zamore, P. D. *Cell* **81**, 171–178 (1995).
- Wickens, M. *Semin. Dev. Biol.* **3**, 399–412 (1992).
- Kobayashi, H. et al. *Cold Spring Harb. Symp. Quant. Biol.* **56**, 437–447 (1991).
- Norbury, C. & Nurse, P. *Annu. Rev. Biochem.* **61**, 441–470 (1992).
- Stewart, D., Shaeffer, J. & Humphrey, M. *Science* **161**, 791–793 (1968).
- Fan, H. & Penman, S. *J. Mol. Biol.* **50**, 665–670 (1970).

10. Wilt, F. H. *Cell* **11**, 673–681 (1977).
11. Sagata, N., Shiokawa, K. & Yamana, K. *Dev. Biol.* **77**, 431–448 (1980).
12. Kanaki, J. P. & Newport, J. W. *Dev. Biol.* **146**, 191–213 (1991).
13. Dambrough, C. & Ford, F. J. *Dev. Biol.* **71**, 323–340 (1979).
14. Pawlowski, P. J. & Rodriguez, L. V. *Dev. Biol.* **40**, 71–77 (1974).
15. Slater, D. W. & Slater, I. *Nature* **240**, 333–337 (1972).
16. Raabe, T., Boilum, F. J. & Manley, J. L. *Nature* **353**, 229–234 (1991).
17. Elledge, S. J. & Harper, J. W. *Curr. Opin. Cell Biol.* **6**, 847–852 (1994).
18. Vesely, J. et al. *Eur. J. Biochem.* **224**, 771–786 (1994).
19. Zhao, W. & Manley, J. L. *Mol. Cell. Biol.* **16**, 2378–2386 (1996).
20. Wahle, E., Martin, G., Schlitz, E. & Keller, W. *EMBO J.* **10**, 4251–4252 (1991).
21. Thuresson, A. et al. *Proc. Natl Acad. Sci. USA* **91**, 979–983 (1994).
22. Ballantyne, S. et al. *RNA* **1**, 64–78 (1995).
23. Bienroth, S., Wahle, E., Suter-Crazolera, C. & Keller, W. *J. Biol. Chem.* **266**, 19768–19776 (1991).
24. Murthy, K. G. K. & Manley, J. L. *J. Biol. Chem.* **267**, 14804–14811 (1992).
25. Gerhart, J., Wu, M. & Kirschner, M. J. *Cell. Biol.* **98**, 1247–1255 (1984).
26. Manley, J. L. et al. *Proc. Natl Acad. Sci. USA* **77**, 3855–3859 (1980).
27. Wang, Y. & Prives, C. *Nature* **376**, 88–91 (1995).
28. Prives, C. & Foukal, D. *Meth. Cell Biol.* **36**, 185–210 (1991).
29. Martin, G. & Keller, W. *EMBO J.* **15**, 2593–2603 (1996).
30. Raabe, T., Murthy, K. G. K. & Manley, J. L. *Mol. Cell. Biol.* **14**, 2946–2957 (1994).

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Structural basis for the binding of a globular antifreeze protein to ice

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ANTIFREEZE proteins (AFPs) have the unique ability to adsorb to ice and inhibit its growth¹. Many organisms ranging from fish to bacteria use AFPs to retard freezing or lessen the damage incurred upon freezing and thawing^{2–6}. The ice-binding mechanism of the long linear α -helical type I AFPs has been attributed to their regularly spaced polar residues matching the ice lattice along a pyramidal plane^{7,8}. In contrast, it is not known how globular antifreeze proteins such as type III AFP that lack repeating ice-binding residues bind to ice. Here we report the 1.25 Å crystal structure of recombinant type III AFP (QAE isoform⁹) from eel pout (*Macrozoarces americanus*), which reveals a remarkably flat amphipathic ice-binding site where five hydrogen-bonding atoms match two ranks of oxygens on the {1010} ice prism plane in the <0001> direction, giving high ice-binding affinity and specificity. This binding site, substantiated by the structures and properties of several ice-binding site mutants, suggests that the AFP occupies a niche in the ice surface in which it covers the basal plane while binding to the prism face.

The crystal structure has been solved by multiple isomorphous replacement and refined to 1.25 Å resolution (Table 1). In addition, the structures of five activity mutants have been determined. The protein is a compact, angular structure (Fig. 1) in which the overall fold comprises numerous short and imperfect β -strands and one turn of α -helix (Fig. 1a). One of its unique features is that there are many ' β -structure-like' interstrand main-chain hydrogen bonds that give the protein a rigid, globular fold (Fig. 1b). The ice-binding residues previously identified by site-directed mutagenesis^{10,11}, namely Gln 9, Thr 18, Gln 44 and Asn 14, are clustered

together on one side of the protein, opposite to the N and C termini. The side-chain O atoms of Gln 9, Thr 15 and Thr 18, the N atom of Gln 44, and the main-chain O atom of Ala 16 form a flat surface (Fig. 1b) with the largest deviation being the carbonyl of Ala 16 (0.31 Å to the ideal plane). This remarkably flat plane, which is rather uncommon in a small protein, is surrounded by the neighbouring hydrophobic residues (Leu 10, Pro 12, Ile 13, Leu 19, Val 20, Val 41 and Leu 51). Thr 18, Ala 16 carbonyl, and Gln 44 line up on one side of a pseudo-parallellogram, with Gln 9 and Thr 15 occupying the parallel side 4.5 Å apart. The side chain of Met 21 emerges into the centre of this plane and is well positioned to stabilize the side chains of Gln 9, Thr 15, Thr 18 and the Ala 16 carbonyl by van der Waals interactions. Furthermore, Gln 44 and Asn 14 are stabilized by hydrogen bonds to the side chain of Lys 61. Consequently, it appears that there are constraints to the side-chain flexibility of all the ice-binding residues.

The interatomic distances between five key atoms of the ice-binding residues form a close match to the spacing of water O

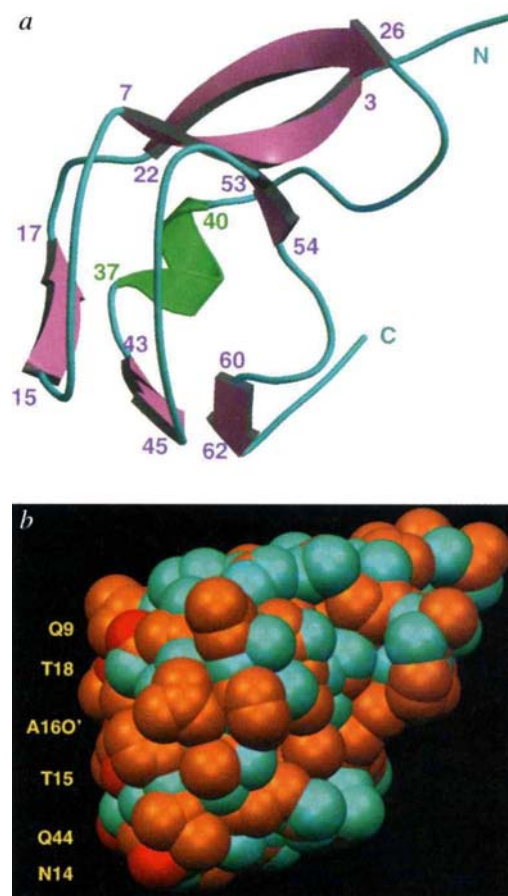


FIG. 1 a, Ribbon diagram of the AFP, with secondary-structure elements labelled by amino-acid number. Secondary structures were assigned using STRIDE²¹ (but using strict hydrogen-bonding criteria, no or little β -structure can be identified). The overall topology is similar to the NMR structure²², although with less well-defined β -sheets. The short two-stranded β -sheet²² is no longer apparent and the 'orthogonal' arrangement of the two triple β -sheets is less pronounced in the X-ray structure. In addition, one turn of α -helix is found in the crystal structure, which is in agreement with the further refined NMR model (F. Sönnichsen, personal communication). The diagram was generated using Molscript²³ and Raster3D²⁴. b, Space-filling plot illustrating the flat ice-binding surface plane and the angularity between the ice-binding plane and surface planes on the top and bottom of the protein. The orientation of the AFP is similar to that in Fig. 1a and in Fig. 2a; the ice-binding residues described in the text are coloured red. This diagram and Fig. 2 were produced using SETOR²⁵.

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TABLE 1 Structure determination and refinement

Data and phasing statistics							
Data set	Resolution (Å)	Total reflections	Unique reflections	Completeness (%)	R_{sym}	R_{iso}	$\langle F_H \rangle / \langle E \rangle$
Native 1	2.5	6,111	2,186	96.5	0.060		
K ₂ Pt(CN) ₄	2.5	13,484	2,187	96.1	0.057	0.131	1.33
Seleno-Met	2.5	10,978	2,325	99.5	0.054	0.196	1.91
Seleno-Met/Pt	2.5	13,588	2,245	96.1	0.114	0.261	1.75
Overall mean figure of merit at 2.5 Å resolution: 0.71							
Native 2	1.25	46,873	16,529	96.6	0.049		
A16H mutant	1.43	40,197	9,650	83.9	0.065		
A16T mutant	1.6	28,480	8,075	95.2	0.059		
A16C mutant	1.6	19,644	7,177	84.5	0.062		
T18N mutant	2.0	13,116	4,088	91.7	0.069		
N14S/Q44T double mutant	2.6	3,195	1,720	82.2	0.073		
Refinement statistics							
Data set	Native 2	A16H	A16T	A16C	T18N	N14S/Q44T	
Unit cell <i>a</i> (Å)	32.33	33.30	33.27	32.65	32.67	32.65	
<i>b</i> (Å)	38.98	39.93	40.11	39.10	38.91	39.08	
<i>c</i> (Å)	45.48	44.66	44.86	46.26	46.35	47.07	
Resolution range (Å)	8.0–1.25	8.0–1.45	8.0–1.6	8.0–1.6	8.0–2.0	8.0–2.6	
R_{factor} (R_{free} , 5%)	0.198 (0.225)	0.191 (0.250)	0.199 (0.226)	0.194 (0.268)	0.154 (0.253)	0.146 (0.241)	
Number of protein atoms	482	490	484	483	483	478	
Number of water atoms	90	70	72	70	39	32	
R.m.s. deviation from ideal geometry							
Bond lengths (Å)	0.014	0.014	0.015	0.013	0.015	0.014	
Bond angles (°)	2.94	2.77	2.82	2.92	3.12	3.38	

Space group: $P2_12_12_1$. $R_{\text{sym}} = \sum |I - \langle I \rangle| / \sum \langle I \rangle$; $R_{\text{iso}} = \sum \|F_{\text{PH}} - |F_{\text{P}}|\| / \sum |F_{\text{P}}|$; phasing power ($\langle F_H \rangle / \langle E \rangle$) is the r.m.s. value of F_H divided by the r.m.s. lack of closure error; overall figure of merit is the cosine of the mean phase error. I is the diffraction intensity and F_{P} , F_{PH} and F_{H} are the native, derivative and heavy-atom structure factors, respectively.

atoms in the prism plane. Manual docking shows that the five protein atoms in their current positions in the X-ray structure can simultaneously form hydrogen bonds to the water O atoms on the $\{10\bar{1}0\}$ plane along the $\langle 0001 \rangle$ direction (Fig. 2). The hydrogen-bond lengths range from 2.7 to 3.1 Å, of which Thr 15 and Thr 18 provide the shortest interactions and Ala 16 carbonyl the longest. There are no residues in the surface patch that would sterically interfere with the interaction. Docking of the ice-binding surface in various directions to other ice lattice planes, including the basal plane $\{0001\}$ and secondary prism planes such as $\{1\bar{2}10\}$, failed to give rise to a reasonable distance match that would allow all five ice-binding atoms simultaneously to hydrogen-bond to the chosen ice plane. Our results agree with previous ice-etching studies which showed that type III AFP binds to the prism plane $\{1010\}$ of the hexagonal ice lattice¹².

Asn 14, arguably the most important ice-binding residue (the protein in which Asn 14 is substituted by Ser, has only 25% activity¹¹), does not lie in the ice-binding plane, in spite of its close proximity (Fig. 2b). In the docking model, the side chain of Asn 14 forms a hydrogen bond (2.7 Å) to an advanced ice water molecule in the conjunction or hinge region between the prism and basal planes (Fig. 2b). Therefore the complete binding plane is in fact a higher-index plane that is very close to the prism face. It appears that Asn 14 not only contributes to the overall ice-binding affinity, but also plays a pivotal role in initiating binding at transition points between prism and basal planes. The interaction could occur at the rounded edges of the prism surfaces or on the basal plane. In the latter situation we envisage that the AFP binds very weakly (through Asn 14) at the leading edge of a new hexagonal ice layer on the basal plane, thereby slowing its lateral expansion. Frequently, a second layer of ice would catch up with the lower layer to make contact with a second ice-binding residue (Gln 44). This, and additional contacts to subsequent ice layers, would further slow the dissociation of the protein. At these intermediate stages, AFP binding to ice is still reversible and

similar to that of ice-binding site mutants, which have the ability to slow and shape the growth of ice but not prevent it¹⁰ (Fig. 3). This model implies that the AFP shapes the site on ice to which it binds. Eventually the protein–ice contact site is fully formed and AFP binding is virtually irreversible.

The essential features of the ice-binding site are supported by the results of mutagenesis. One prediction of the model was that Thr 15 should be an ice-binding residue, which was borne out when the Thr 15 → Ala mutant was shown to have only ~70% of the activity of wild-type AFP. Typically, the loss of a single hydrogen-bonding group caused by shortening the side chain as in Gln 9 → Thr¹⁰, Thr 15 → Ala and Thr 18 → Ala decreased antifreeze activity to 70–80%. Similar mutations in the conjunction area had more severe effects (Gln 44 → Thr, 50% activity; Asn 14 → Ser (ref. 11)). Steric interference and the consequent loss of multiple hydrogen bonds had a predictably serious impact on ice binding. Ala 16 replacements formed a series of increasingly deleterious mutations, ranging from Ala 16 → Cys (100% activity) to Ala 16 → His (25% activity) (C.I.D. *et al.*, unpublished results). Structures for several of these mutants (Table 1) have provided evidence that the bulky side chain of His 16 and the branched side chain of Thr 16 (75% activity), which are positioned immediately adjacent to the ice-binding site, directly interfere with ice binding through van der Waals repulsion. Lengthening the side chain of an ice-binding site residue had a drastic effect (Thr 18 → Asn, 10% activity¹¹). The structure of the Thr 18 → Asn mutant clearly shows that the longer side chain of Asn 18 projects above the surface. van der Waals repulsion resulting from the Asn 18 side chain would prevent at least Gln 9 and the Ala 16 carbonyl from forming hydrogen bonds with the ice lattice.

Ice-binding site mutants of type III AFP not only have decreased antifreeze activity, but also drastically influence the morphology of the ice crystals¹⁰. These crystals vary from the stable, short hexagonal bipyramid formed in the presence of wild-

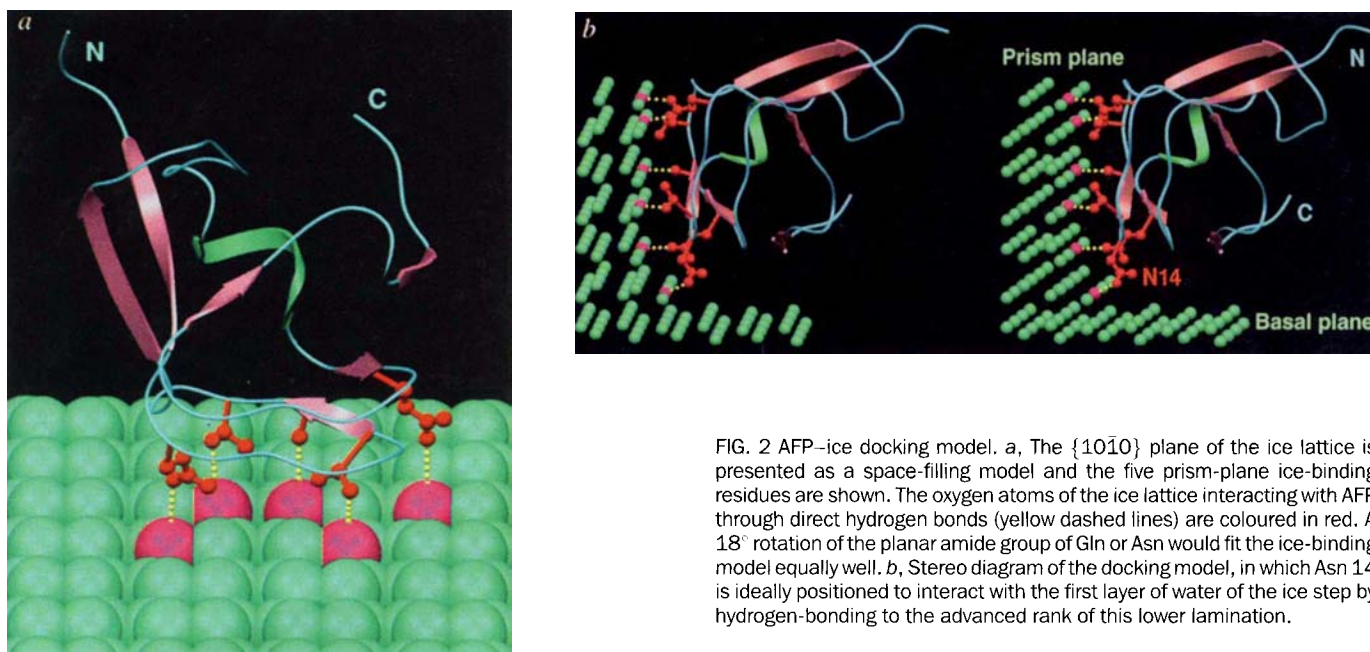


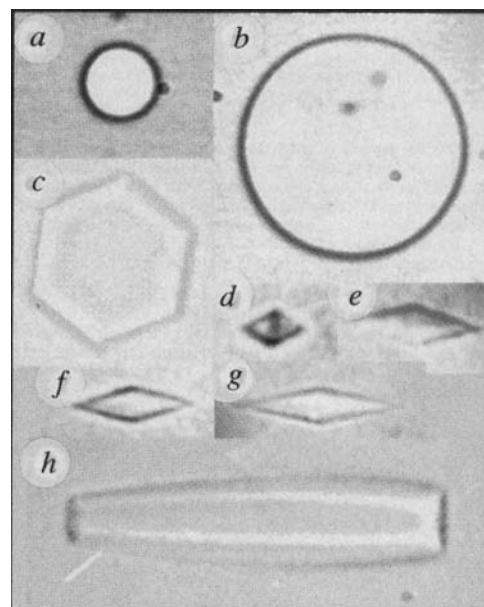
FIG. 2 AFP-ice docking model. *a*, The $\{10\bar{1}0\}$ plane of the ice lattice is presented as a space-filling model and the five prism-plane ice-binding residues are shown. The oxygen atoms of the ice lattice interacting with AFP through direct hydrogen bonds (yellow dashed lines) are coloured in red. A 18° rotation of the planar amide group of Gln or Asn would fit the ice-binding model equally well. *b*, Stereo diagram of the docking model, in which Asn 14 is ideally positioned to interact with the first layer of water of the ice step by hydrogen-bonding to the advanced rank of this lower lamination.

type AFP, to the elongated bipyramids which develop in the presence of mutants and lengthen along the *c*-axis with time (Fig. 3). Generally, the less activity a mutant possesses, the longer the ice crystal becomes. This can be attributed to the reduced affinity between the mutant AFP and the ice lattice, which would result in more growth of prism fronts, giving rise to a crystal elongated along the *c*-axis¹⁰. The double mutant (Asn 14 $\rightarrow$ Ser/Gln 44 $\rightarrow$ Thr) (10% activity) shows an extreme expression of this phenotype, with a smoothly contoured surface and lack of angularity at the pyramidal junction (Fig. 3). As Asn 14 and Gln 44 are both in the conjunction region, this phenotype can be attributed to this mutant's reduced ability to initiate binding.

Elucidation of the structural basis for high-affinity ice binding and the mechanism of action of globular type III AFP was made possible by acquisition of a high-resolution X-ray structure and analysis of site-specific mutations. In addition to the high binding affinity and geometry specificity provided by the precise matching

of multiple hydrogen bonds of hydrophilic residues, hydrophobic residues may also help shield the hydrogen bonds and even participate in van der Waals interaction with ice because of the excellent shape complementarity between the flat protein surface and flat ice lattice. As has been pointed out for type I AFP⁴, the flatness of the ice-binding site of type III AFP is a crucial feature of the ice-binding mechanism. One significant difference between these two AFPs is that their mechanisms of action are determined by their affinity for different ice lattice planes. Whereas type I AFP can constrain ice to form a hexagonal bipyramid by virtue of its binding to an ice pyramidal plane¹³, a small globular protein like type III AFP which binds to the ice-prism plane can form a hexagonal bipyramid only by occupying a step in the ice surface (Fig. 2*b*). This may be facilitated by the non-planar residue Asn 14 making the initial protein-ice interaction at the junction site. The angularity of the protein is ideal for occupancy of a step-like niche in ice. This property, together with the flatness and spatial geometry of the ice-binding site, are structural requirements

FIG. 3 Photomicrographs of ice crystals. In *a*–*c*, the *c*-axis is perpendicular to the paper; *d*–*h*, the *c*-axis is approximately horizontal. An ice crystal (*a*) in the absence of AFP grows as a rounded disk. At slight undercooling, water joins the ice lattice normal to the *c*-axis at a rate that is $\sim 100\times$ greater than addition along the *c*-axis²⁶. *b*, Growth after 5 s from *a*. Wild-type AFP at low concentrations (μM), or activity mutants such as Thr 18 $\rightarrow$ Asn, shape the disc into a hexagonal plate (*c*) because the AFP preferentially binds to and inhibits growth on the prism surfaces. At higher AFP concentrations, growth normal to the *c*-axis is further retarded such that the ice crystal is shaped into a hexagonal bipyramid (*d*). Mutants Gln 9 $\rightarrow$ Thr (*e*) and Thr 15 $\rightarrow$ Ala (*f*), produce hexagonal bipyramids that are more elongated. *g*, Growth after 10 min from (*f*); ice crystals further elongate, which can be attributed to the filling in or overgrowth of ice niches occupied by AFP. One extreme expression (*h*) of this is the double mutant (Asn 14 $\rightarrow$ Ser/Gln 44 $\rightarrow$ Thr). It has little ability to retard the lateral expansion of new ice sheets emerging from the basal planes as the residues that bind at the transition between prism and basal planes have been mutated.



that exclude most globular proteins from binding to ice. □

Methods

The crystallization of type III antifreeze protein and preparation of its platinum derivative have been described¹⁴. Extensive attempts to obtain a second derivative using wild-type AFP, various Cys and His mutants either through co-crystallization or soaking experiments, failed. Therefore, type III AFP was labelled with selenomethionine¹⁵ and crystallized under similar conditions to the wild-type protein, although at lower pH (3.25 rather than 4.50). The 1.25 Å-resolution data were collected at beam X11 ($\lambda = 0.91$ Å), DESY, EMBL Hamburg Outstation. All other data were collected using a Mar Research imaging plate equipped with an Rigaku rotating-anode generator. Data were processed using DENZO¹⁶ and CCP4¹⁷. The difference Patterson map of the seleno-methionyl protein was not interpretable, nor was the difference map cross-phased by the Pt derivative. The direct method¹⁸, however, enabled all five internal Se atom positions to be determined. The MIRAS phases calculated with MLPHARE¹⁹ were improved by solvent flattening and skeletonization using CCP4¹⁷, which resulted in a readily interpretable 2.5 Å map. Refinement was performed using X-PLOR²⁰. All data were used (no σ cut) in the refinement, in which 5% randomly chosen data were set aside for R_{free} calculation. All residues are in the allowed Ramchandran regions and all side chains are visible, except for one or two N-terminal residues. Multiple rotamers were observed for residues 2, 24, 25 and 55 in the native model, but not for any of the ice-binding residues. Further refinement involving anisotropic temperature factor and hydrogen atoms for the native protein model is in progress.

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1. Raymond, J. A. & DeVries, A. L. *Proc. Natl Acad. Sci. USA* **74**, 2589–2593 (1977).
2. Davies, P. L. & Hew, C. L. *FASEB J.* **4**, 2460–2468 (1990).
3. Duman, J. G., Xu, L., Neven, L. G., Tursmon, D. & Wu, D. W. in *Insects at Low Temperatures* (eds Lee, R. E. & Denlinger, D. L.) 94–127 (Chapman & Hall, New York, 1991).
4. Griffith, M., Ala, P., Yang, D. S. C., Hon, W. C. & Moffat, B. A. *Plant Physiol.* **100**, 593–596 (1992).
5. Urrutia, M. E., Duman, J. G. & Knight, C. A. *Biochim Biophys. Acta* **1121**, 199–206 (1992).
6. Sun, X., Griffith, M., Pasternak, J. J. & Glick, B. R. *Can. J. Microbiol.* **41**, 776–784 (1995).
7. Knight, C. A., Cheng, C. C. & DeVries, A. L. *Biophys. J.* **59**, 409–418 (1991).
8. Sichen, F. & Yang, D. S. C. *Nature* **375**, 427–431 (1995).
9. Chao, H., Davies, P. L., Sykes, B. D. & Sönnichsen, F. D. *Prot. Sci.* **2**, 1411–1428 (1993).
10. Chao, H. thesis, Queen's Univ. (1994).
11. Chao, H., Sönnichsen, F. D., DeLuca, C. I., Sykes, B. D. & Davies, P. L. *Prot. Sci.* **3**, 1760–1769 (1994).
12. Cheng, C. C. & DeVries, A. L. in *Life Under Extreme Conditions* (ed. di Prisco, G.) 1–14 (Springer, Berlin, 1991).
13. Wen, D. & Laursen, R. A. *Biophys. J.* **63**, 1659–1662 (1992).
14. Jia, Z., DeLuca, C. I. & Davies, P. L. *Prot. Sci.* **4**, 1236–1238 (1995).
15. Hendrickson, W. A., Horton, J. R. & LeMaster, D. M. *EMBO J.* **9**, 1665–1672 (1990).
16. Otwinowski, Z. in *Data Collection and Processing* (eds Sawyer, L., Issacs, N. & Bailey, S.) 56–62 (SERC Daresbury Laboratory, Warrington, UK, 1993).
17. CCP4 Acta Crystallogr. **D50**, 760–763 (1994).
18. Sheldrick, G. M. *SHELXS86. Program for the Solution of Crystal Structures* (Univ. of Göttingen, Germany, 1986).
19. Otwinowski, Z. in *Isomorphous Replacement and Anomalous Scattering* (eds Sawyer, L., Issacs, N. & Bailey, S.) 80–86 (SERC Daresbury Laboratory, Warrington, UK, 1991).
20. Brünger, A. T. *X-PLOR, Version 3.1. A System for X-ray Crystallography and NMR* (Yale Univ. Press, New Haven, 1992).
21. Frishman, D. & Argos, P. *Prot. Struct. Funct. Genet.* **23**, 566–579 (1995).
22. Sönnichsen, F. D., Sykes, B. D., Chao, H. & Davies, P. L. *Science* **259**, 1154–1157 (1993).
23. Kraulis, P. J. *J. Appl. Crystallogr.* **24**, 946–950 (1991).
24. Merrit, E. A. & Murphy, M. E. P. *Acta Crystallogr.* **D50**, 869–873 (1994).
25. Evans, S. V. *J. Mol. Graph.* **11**, 134–138 (1993).
26. Kallungal, J. P. thesis, Syracuse Univ. (1975).

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CORRESPONDENCE and requests for materials should be addressed to Z.J. (e-mail: jia@crystal.biochem.queensu.ca). The coordinates of type III AFP have been deposited in the Brookhaven Protein Data Bank, accession code 1MSI. For an animation of AFP binding to ice, visit the World-Wide Web site: <http://crystal.biochem.queensu.ca/nature>.

ERRATA

The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1

Estelle Oberlin, Ali Amara, Françoise Bachelierie, Christine Bessia, Jean-Louis Virelizier, Fernando Arenzana-Seisdedos, Olivier Schwartz, Jean-Michel Heard, Ian Clark-Lewis, Daniel F. Legler, Marcel Loetscher, Marco Baggiolini & Bernhard Moser

Nature **382**, 833–835 (1996)

THE labelling of the two lower panels of Fig. 2b was accidentally omitted. These panels showed inhibition (left panel) or lack of inhibition (right panel) of syncytia formation by 100 nM SDF-1 or RANTES, respectively. The labels should thus have read 'HIV-1_{LAV} + SDF-1' (left) and 'HIV-1_{LAV} + RANTES' (right). □

Specific cytotoxic T cells eliminate cells producing neutralizing antibodies

Oliver Planz, Peter Seiler, Hans Hengartner & Rolf M. Zinkernagel

Nature **382**, 726–729 (1996)

THE title shown above is incomplete and should read "Specific cytotoxic T cells eliminate B cells producing virus-neutralizing antibodies". □

ADDENDUM

Three-dimensional structure of human cytomegalovirus protease

Huey-Sheng Shieh, Ravi G. Kurumbail, Anna M. Stevens, Roderick A. Stegeman, Eric J. Sturman, Jina Y. Pak, Arthur J. Wittwer, Mark O. Palmier, Roger C. Wiegand, Barry C. Holwerda & William C. Stallings

Nature **383**, 279–282 (1996)

This letter contains six additional figures as Supplementary Information. These are available on *Nature's* World-Wide Web site (<http://www.nature.com>) or as paper copy from Mary Sheehan at the London editorial office of *Nature*. □

Gadgets for the lab that has everything

New on the market has holiday gift ideas for the colleague who has everything, including a geographic information system, a Raman imaging microscope, a phase-shifting interferometer and software for predicting crystal morphologies.

MAPLE V release 4.0 for the Macintosh is the new version of the **mathematical computational system** that is now available from Waterloo in the UK. The new version demonstrates mathematical problem-solving and visualization, as well as its symbolic problem-solving algorithms. This program is designed to use algebraic expression with symbolic variables and return answers as mathematical objects. The Power Macintosh version includes a native executable that delivers increased processing speed, as well as continued support for 68K machines with and without floating-point units (FPUs). The algorithms have been extended to include a set of routines for dealing with piecewise defined functions, an improved equation solver to handle inequities and inverse trigonometric functions, and the ability to deal with two-dimensional geometry. The program's interface has been redesigned to provide a processing environment for technical documents. There are new features such as hyperlinks, collapsible outlining and typeset mathematics in input, output and text regions, allowing the user to produce high-quality printed and electronic documents.

Reader Enquiry 100

Field and lab

Workstation Source has introduced the TexasMicro PC, a rugged **hard-body, handheld personal computer**. The unit weighs approximately 1.4 kg and can easily be carried to locations previously inaccessible to a high-performance computer. It is designed to be a rugged performer and can operate despite rough handling and harsh environments, according to the manufacturer. Continuous operation is possible without changing the battery for up to eight hours, minimizing the need for battery swapping. Batteries can also be 'hot swapped' while the unit is switched on. A standard PC architecture is used, running all DOS and Windows programs unmodified. Application software packages are said to allow rapid system deployment. Many PC-card interface options are available for interfacing the computer to the outside world. This allows custom tailoring of the system to a specific application using optional features, such as the global positioning system (GPS), spread spectrum wireless local area network (LAN) and wide area (WAN) communi-



Workstation Source's handheld PC.

cations, as well as removable memory and an on-line camera. The unit has a 75-MHz 80486DX4 CPU and up to 32 MB of memory, allowing it to run sophisticated graphics programs. Data and programs are stored on a rugged built-in, high-capacity hard disk. The transfective VGA liquid-crystal display provides readability in direct sunlight or in a dark environment, using the internal backlighting. A high-resolution touch-screen makes data entry using character recognition simple. A tethered pen eliminates the possibility of losing input capability.

Reader Enquiry 101

The Institute of Terrestrial Ecology has released Ranges V software, sponsored by the Norwegian Institute for Nature Research, the US Fish and Wildlife Service (new National Biological Service) and Greenfalk. Ranges V is a specialized **geographic information system** for ecological data, designed primarily to process the large quantities of data generated by radio-tracking. It calculates distances, headings, times and speeds between locations, area statistics for sets of locations and interactions between location sets or within habitat maps of vector and raster data (for example, from scanning satellites). Ranges V is both a planning aid, with autocorrelation and area-increment analyses to optimize data collection for home-range assessment, and a processing system for dispersal detection, sociality scoring and range estimation by polygon techniques (peeled and concave), cluster analysis, bivariate kernels and contouring (harmonic mean and other kernels, also with weighted cores and least squares cross validation). High data volumes, previously a serious problem for many radio-tag studies, are accommodated by multi-set files and batch operations. There is digitizer support for mapping, porting of

files in DXF, bytemap and ARC/INFOgrid ASCII formats, as well as a spreadsheet interchange of raw data or output of statistics as text. Although intended primarily for zoologists, Ranges V should prove to be generally useful for digitizing maps to record coordinates, for estimating areas, peripheries and overlaps of shapes, and for generating contours of plant density and distance distributions. It is available now for MS-DOS/Windows and Acorn RISC systems; future development will focus on an expert analysis system, Ranges VI.

Reader Enquiry 102

The universal **portable datalogger** now available from E. Clark features four analog ports and one digital port for input, which accept a wide range of available signals, including voltage, current, frequency and electrical resistance signals. Programmable connection plugs allow the user to connect to virtually any sensor and display/catalogue with programmable zero-adjust, scaling factors and engineering units. A wide variety of standard sensors is available, including temperature, humidity, pressure and air velocity. There is sufficient memory capacity for 25,000 measurements, with an available



Universal analog and digital datalogger.

100,000 measurement option. The 2290-8 is a full-featured datalogger, including programmable date and time, as well as callable maximum, minimum and average values. Datalogging can be activated manually by preprogrammed start/stop dates and times, or by using limit-value overshoot. The frequency of measurement logging is user-programmable from every 1 s to every 60 h. The unit includes analog and RS232 output ports, it can connect directly to a printer or computer for easy downloading of information and

has the capability to network multiple units together. High resolution is achieved through a 16-bit A/D converter.
Reader Enquiry 103

Physical sciences

Oxford Materials has announced the release of MetaMorph, a software package for **predicting and visualizing crystal morphologies**. Morphology prediction is often vital in chemical synthesis, where control of particle size and shape is required to improve particle handling and flow. This technology uses calculated surface energies to produce three-dimensional crystal shapes, and interactive visualization allows the user to see the effect of changing surface energies on the crystal morphology. It is designed to work with the company's CrysAlis software package for simulating defects and surfaces in inorganic materials. Predictions from MetaMorph, using surface energies from CrysAlis, have been shown to be in agreement with real morphologies for complex structures like $\alpha\text{-Al}_2\text{O}_3$ and $\alpha\text{-Fe}_2\text{O}_3$, where surface rumpling and relaxation effects are important. This program is supported on Silicon Graphics workstations, and customers can opt for one of two schemes to access the software — outright purchase of a right-to-use licence or, alternatively, annual lease inclusive of hotline support and software upgrades. Trial evaluation leases can be arranged.

Reader Enquiry 104

Berkeley Lab researchers have discovered a way to **incorporate phase shifting into point-diffraction interferometry** (PDI), which may significantly enhance PDI-based measurement systems and optical component testing devices. This new point-diffraction interferometer, called a phase-shifting interferometer (PSI), uses the reference beam more efficiently to create fringe interference patterns, or interferograms, of the test and reference beams. Efficiency is increased by a stated 100–1,000 times over conventional point-diffraction interferometers. With millimetre-sized components it can measure optics of the order of metres. This system offers the user phase-shift capability and is said to increase the resolution of the fringe of the recording surface to improve measurement capability. It is said to make PDI applicable over a range of wavelengths from microwave to x-ray devices, to maintain a higher intensity reference wave and greatly increase fringe visibility. Moreover, it efficiently uses source radiation, attenuating the test wave by only one order of magnitude (3–4 orders of magnitude are not uncommon with current PDI systems, according to the manufacturer). The PDI eliminates the need to use a semi-transparent mem-

brane mask; these masks are fragile, difficult to construct and are frequently damaged or contaminated with use. Most components for this system (gratings, pin-hole apertures, mirrors) are available off the shelf. The Berkeley Lab is seeking companies that are interested in licensing this technology and/or entering collaborative arrangements for its further development.

Reader Enquiry 105

Now available from Glen Spectra are **filters for astronomy**, including filters manufactured by Omega Optical. Standard filter sets are available, as are custom-made filters, ranging from the deep ultraviolet to the mid-infrared. The company can fill filter needs from a standard filter set that isolates broad areas of the optical spectrum to custom-made filters. Peak wavelengths range from the deep ultraviolet to the mid-infrared, with bandwidths ranging from 0.15 nm to several hundred nanometres. These astronomy filters are provided with AR coatings and are of imaging quality.

Reader Enquiry 106

Microscopy

The Techne MTS-1 **thermal microscope stage** is a precision instrument designed to suit most makes of microscopes and offers the user a cool or warm stage for operation over the temperature range of 4 °C to 50 °C. Although primarily for use with microscopes fitted with micromanipulators, the stage is also suitable for cell and embryo research, including transgenic applications, the study of spermatozoa, amoeba, ova and cell cultures. Additionally, the MTS-1 can be supplied without a stage, but with piped connections, making the unit suitable for use with spec-



Techne's warm and cool thermal stage.

trophotometers, fluorometers and other applications where a small flow of temperature-controlled water is needed to maintain the temperature of the sample or sample holder. Stages are currently available for use with micromanipulators, 35-mm Petri dishes and microscope slides. The temperature is set on an analogue scale with a sensor in the thermal stage giving a digital readout of the working temperature. The main unit is a self-contained, peltier-cooled chamber, which has a small reservoir of liquid that is pumped in a closed circuit through to the thermal stage giving precision control where it is needed.

Reader Enquiry 107

The TopoMetrix Explorer **modular microscope** has been designed to incorporate features available in a scanning probe microscope (SPM). The modular design allows flexibility in all scanning modes and image analysis. In a new brochure, the manufacturer describes the features of this system, which allows the atomic resolution of molybdenum disulphide and the utility of scan linearity obtained using the TrueMetrix feature. Current specifications are described to give the prospective user a simple means of comparison between SPM systems. These examples and other product and application developments may be found on the Internet at www.topometrix.com.

Reader Enquiry 108

The Renishaw **Raman imaging microscope** features high spatial resolution (1 μm) and high sensitivity (>30 per cent efficiency), bringing improved analytical capability to a Raman instrument, according to the manufacturer. The company has introduced direct two-dimensional Raman imaging, Raman mapping, line focusing and remote capabilities using fibre optics in one compact instrument. The microscope can be used in a confocal configuration to provide 2- μm depth-of-field imaging from transparent samples. The 'extended-scan' facility of the instrument provides the benefit of both Raman spectra and fluorescence data, over the full spectral range (to 1.0 μm) on the same instrument without any system modification. The instrument has been used in a variety of fields, including semiconductors, pharmaceuticals, corrosion science, quality assurance/quality control, forensic science, hard coating (diamond, tin, and the like), mineralogy, gemology and biomedicine. Recent work in the latter of these application areas is said to demonstrate the capability of Raman imaging for such applications as determining the age of living bovine ovary cells and the development of Raman spectroscopy as a mechanistic tool for epidural procedures.

Reader Enquiry 109

Meridian Instruments has introduced a new addition to its family of **confocal imaging systems**. The TR series comprises a line of six ergonomically designed instruments based on a single prototype. The series is the first laser-scanning confocal imaging system to be offered by the company that is designed with both an inverted microscope and the optimal efficiency of a single mirror path. With up to five detectors in conjunction with up to four simultaneous excitation lines, the TR series offers improved axial resolution and image quality within a Windows 95-based graphical user interface. This system should find use in simple personal confocal scanning through to highly complex multi-disciplined configurations.

Reader Enquiry 110

Recently released by World Precision Instruments (WPI) is the **MicroCam microscope camera** by Polaroid, which is designed to give high-contrast, full-colour or black-and-white photographs with just the push of a button. According to the manufacturer, the MicroCam is easily placed into the eyepiece or photo tube of virtually any light microscope — then focus and shoot. The camera is said to take mere seconds to produce a self-developing photograph with high



Polaroid's microscope camera from WPI.

resolution and definition. MicroCam also comes with an electronic cable release, which reduces the risk of camera movement and, hence, could cause blurring. Engineered to be ultra-lightweight and compact, the camera can easily be moved from one microscope to another. Moreover, this is an SLR (single-lens reflex) camera; users see the specimen directly through the camera for accurate 'what-you-see-is-what-you-get' images. It also reads the light level, automatically adjusting for precise exposures and

shows each camera function on one easy-to-read multilingual LED display panel.

Reader Enquiry 111

With the Ultra Objective from Surface Imaging Systems, Carl Zeiss provides an improved combination of **classical light microscopy and nanometre-resolution scanning microscopy**. According to the manufacturer, this objective improves the resolution capability of the light microscope, by extending it down to a resolution of one nanometre, allowing micro- and nanostructures to be characterized qualitatively and quantitatively. Classical microscopy offers high operating convenience and productivity and is now enhanced by new types of contrast, such as topography, magnetic and potential contrast. It is possible not only to obtain images of high resolution, but also to perform dimension measurements easily and with high accuracy. Applications of this system range from studying semiconductor structures to coatings, thin films, materials science and the life sciences. Depending on the application, the objective can be combined with different microscopes from Carl Zeiss.

Reader Enquiry 112

Spin control

Eppendorf has designed two new **centrifuges**, the 5810 and the refrigerated version 5810 R, to be extremely compact bench-top devices with an access height of only 28 cm. They can accommodate up to 36 Falcon tubes of 15 ml, or as many as 100 blood-collecting cups. After the rotor has been loaded, the motorized lid closes without requiring any force. There are 34 individual programs available with rotor-speed settings of up to 14,000 r.p.m. The centrifuges are safe and comply with the requirements of the international safety standard IEC 1010-2-020. The 5810 R can cool samples rapidly and reliably in the temperature range between -9°C and 40°C .

In a swing-bucket rotor, temperature-sensitive samples are kept at 4°C , even at maximum speed. Other features of the centrifuges 5810/5810 R include an imbalance sensor, automatic rotor recognition, a short-spin function and user-selectable speed (RCF or radius correction values). There are several different swing-bucket and fixed-angle rotors available. The swing-bucket rotor holds up to $4 \times 250\text{-ml}$ rectangular or microtitre plate buckets, which can be inserted directly; the modular design of

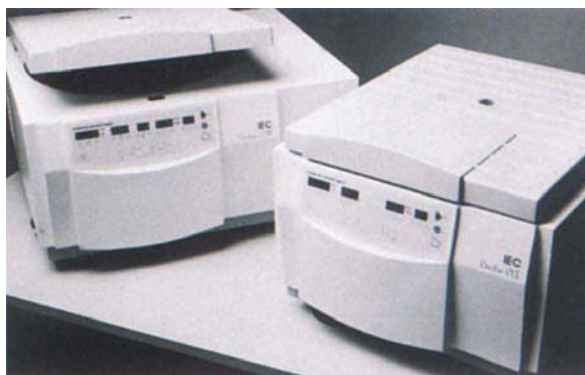


Eppendorf's compact centrifuge.

the rectangular-bucket adapters allows test tubes of different lengths to be used. Furthermore, the rectangular buckets can be sealed with aerosol-tight caps.

Reader Enquiry 113

The Centra-CL3/CL3R (refrigerated) **centrifuges** from IEC are designed to offer quality control over sample preparation. Microprocessor control provides speed control to the nearest 10 r.p.m., selectable temperatures in 1°C increments, automatic calculation of g-force and the ability to store and lock up to 99 protocols. An 'at-speed' timer mode is designed for improved convenience by timing when the unit reaches operating speed. These centrifuges have a low profile, making it easier for technicians to load and unload samples. Moreover, the brushless motor eliminates the need for periodic maintenance and allows for quiet operation. The company has also introduced the new 243 swinging bucket rotor, which accepts sealed aerocarriers, accommodating 0.25-ml to 250-ml sam-



The IEC microprocessor-controlled CL3/CL3R centrifuges.

ples. Exchanging rotors is said to be a snap with a simple, quick-release and quick-mounting mechanism. Additional savings may be realized because the Centra-CL3 will accept rotors from various IEC centrifuges including the recently introduced Centra-CL2.

Reader Enquiry 114

Jouan has announced the introduction of its new family of Autolock **multifunction centrifuges**. The autolock feature is a

rapid rotor mounting system, which ensures that the user has easy access (within a stated 5 s) to a range of eight rotors and 30 inserts and adapters. The B4i and BR4i compact, multifunction centrifuges have been designed to cover a wide range of applications with a capacity of 20 blood tubes, 12 × 15-ml conicals at 3,000g, a 24 × 1.5-ml tube rotor at 18,000g, cyto-centrifugation accessories, sealed microtiter plate and drum rotors. Sealed accessories have been integrated to maintain microbial integrity. The protocols are held in memory with their chosen names clearly written beside the direct recall key. Both centrifuges utilize microprocessor control. In the BR4i, samples are protected by powerful CFC-free refrigeration, which can hold samples at 4 °C when at full speed.

Reader Enquiry 115



Jouan centrifuges.

Labware

Now available from Ocean Optics is a low-cost Windows-interface package that turns Microsoft Excel into a **real-time spectrometer**, using pre-loaded drivers for the company's miniature fibre-optic spectrometers. The company's SS-DLL includes the spectrometer drivers and example programs written in Visual Basic, Borland, Microsoft C/C++ compiler and Microsoft Excel. The link to Excel allows users to acquire and process in real time spectroscopic data, turning their worksheet into a virtual spectrometer. This capability is useful in applications requiring repetitive laboratory measurements, or for time-series measurements for quality control. Moreover, Excel provides graphics, reporting and mathematical analysis, as well as a convenient interface to common LIMS packages. The SS-DLL can be used with the company's PC1000 and ADC-500 card spectrometers, as well as Ocean Optics' spectrometers that interface to the National Instruments DAQ-700 PCMCIA card.

Reader Enquiry 116

The Auto-Spot robot ASP 222 was developed by Abimed Analysen-Technik for the **simultaneous synthesis of individual peptides** on derivatized filter membranes according to the 'spot' method. The robot distributes activated amino acid derivatives onto the filter membrane. Up to 1,600 spots, each corresponding to a single peptide, can be synthesized on four membranes. The ASP 222 should prove

to be a useful screening tool, as sets of peptides can be prepared by an easy-to-use multiple and simultaneous synthesis system. A rack with 44 positions holds the activated amino acid derivatives. The volumes distributed to the synthesis membrane can range from 0.1 to 200 µl. The Windows-based software allows entry of single peptides, as well as the generation of overlapping peptides and sets of analogous sequences. Sequences of peptides and proteins can be entered and processed by the software and handled as standard text files. Reaction times and the number of repeats of a distribution per cycle (up to five) can be specified by the user. A log-file generated by the software documents all synthesis parameters and robotic operations.

Reader Enquiry 117

The PolyScience programmable Liquid-Processor **dispensing system** is capable of dilution, dispensing, pipetting and titration. The rotary axial dispensing mechanism delivers as little as 10 µl through to 1.0 ml with a stated accuracy of 0.1 per cent. A microprocessor-controlled keypad allows parameter choices, such as volume, flow rate, air gaps and flow direction. Multiple programmes can be stored and easily recalled. Moreover, the unit is bottle mounted for convenience and portability, and with the use of adapters it attaches to bottlenecks 25 mm to 45 mm in diameter. The keypad detaches from the bottle for table-top or remote operation. This unit can be activated by handset (supplied), foot switch or PC (RS232). All materials exposed to reagents are composed of chemically resistant PTFE or Al₂O₃.

Reader Enquiry 118

Coast Scientific now offers its new WaterSense **automatic faucet controller**, which is designed to protect against cross-contamination by eliminating fomites and vectors. The device is controlled by an ultrasonic, computer-driven sensor, which automatically controls water flow when the user's hands move under the faucet. This eliminates waste and greatly reduces the risk of cross contamination. No plumbing is required; the unit screws onto the end of the existing faucet and requires one 9-V battery. This product should prove useful in hospitals, laboratories and other locations where contamination is a consideration.

Reader Enquiry 119



PolyScience's new liquid dispenser.

Varian now offers an **ultraviolet, visible and near-infrared data management system**, which has been developed in conjunction with Galactic Industries, the manufacturer of Grams/320. Designed to be easy-to-use, this Windows-based software is said to simplify the setup and management of ultraviolet, visible and near-infrared data sets. This system should prove useful for laboratories that generate large quantities of data; the Spectral Notebook data management system allows users to manage Cary data easily, as well as data from other instruments, and to share this information with colleagues down the hall or around the world. Spectra search-and-match capabilities are available at the click of the mouse. The software also takes advantage of Grams' graphical and maths-processing capability, its library-search option and its chemometric calculations, such as partial least squares and principal component analysis.

Reader Enquiry 120

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

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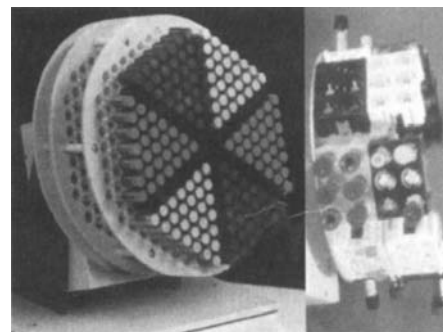
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